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DECLARATION

I, the undersigned, Dr John Chaddock, of Health Protection Agency, do solemnly and sincerely declare that I am an inventor of US patent application No: 09/529,130.

As evidenced by the abridged version of my *curriculum vitae* (Annex 1), I have been actively undertaking research in the technical field of protein chemistry for the last 16 years. I am therefore entirely familiar with protein conjugation chemistry.

I also confirm that I am familiar with the prosecution history (to date) of US 09/529,130.

The methodology involved in coupling two protein molecules (A and B) together is simple, and is achieved through the use of a cross-linking agent (also known as a chemical coupling agent). For example, molecules A and B are separately contacted with a cross-linking agent, which chemically modifies a specific surface group on each of molecules A and B thereby forming derivatised molecules A' and B'. The modified surface group on molecule A' is capable of covalently bonding with the modified surface group on molecule B'. Thus, the coupling reaction is completed by mixing together the two protein molecules A' and B'.

Chemical coupling agents have been commercially available for many years, in particular, well before the priority date (8 October 1997) of the present application. Moreover, the use of cross-linking agents has been widespread for many years (certainly prior to 8 October 1997).

Example 1 of US 09/529,130 illustrates the use of one such coupling agent, namely SPDP, to chemically couple two protein molecules (a galactose-binding lectin, and the LH_N of botulinum neurotoxin). The two molecules are separately contacted with SPDP, and then mixed together to allow covalent conjugation.

In addition, lines 5-7 on page 8 of WO99/17806 (from which US 09/529,130 is derived) confirm that the claimed agent can be produced according to WO96/33273 - an earlier patent application for which I am a named inventor. This confirmation is repeated at lines 16-18 on page 9 of WO99/17806 which explicitly states that "[t]hese and other lectins of the sub-class of galactose-binding lectins can be used as targeting moieties (TM) for conjugates of the type described in WO96/33273."

The conjugates described in the Examples of WO96/33273 confirm that PDPH/EDAC, or Traut's reagent may be employed as an alternative chemical coupling agent to SPDP. Moreover, referring to the specification of WO96/33273 (see the paragraph that follows the Examples and immediately precedes Table 1), it is clear that:-

> *"**any other** coupling chemistry capable of covalently attaching the TM component of the agent to [the] clostridial neurotoxin derived component and known to those of skill in the art is covered by the scope of this application"* [emphasis added].

WO99/17806 (from which US 09/529,130 is derived) also describes at page 9, lines 28-31, using any coupling chemistry capable of attaching the lectin component to the LH_N component of the claimed agents: "Thus, in one embodiment of the invention a galactose-binding lectin is conjugated, using linkages that may include one or more spacer regions, to a derivative of the costridial neurotoxins." WO99/17806 also states at page 10, lines 15-19 that the "TM, L or LH_N and translocation domain components may be separately expressed in a recombinant form and subsequently linked, covalently or non-covalently, to provide the desired agent."

Hence, the skilled artisan would recognize that US 09/529,130 generically describes covalently linking certain lectins to LH_N. Moreover, as further described below, the skilled artisan at the time of filing US 09/529,130 was familiar with chemical coupling agents and their use for covalently linking proteins. Thus, the skilled artisan would also have recognized that the generic description of covalent linkages that may include one or more spacer regions is a well known class of compounds useful for chemically coupling proteins.

SPDP was illustrated as the cross-linking agent in Example 1 of US 09/529,130 simply because it was (and still is) a popular and well-documented coupling agent in the technical field of protein conjugation chemistry. Thus, SPDP is simply one example of a well known class of compounds that may be employed to covalently link together the Targeting Moiety component and the clostridial neurotoxin component of the conjugate described in US 09/529,130. For example, I have also employed SMPB, SMCC (succinimidyl 4-(*N*-maleimidomethyl) cyclohexan-1-carboxylate), and Traut's reagent as alternative chemical coupling agents to link together a galactose-binding lectin or an N-acetylgalactosamine lectin and LH_N.

When preparing the specification of US 09/529,130 it was not considered necessary to provide, in the specification, an exhaustive list of chemical coupling agents. The simple explanation for this is that the use of conventional coupling means for joining together two components of the defined agent was not (and still is not) considered an essential feature of the described invention.

In more detail, commercially available members of the well known coupling agents may be used for conjugation purposes to produce an agent of the invention described in US09/529,130. By way of example, I refer to the following pre-1997 publications:-

Annex 2	- Hermanson, G.T. (1996), Bioconjugate techniques, Academic Press;
Annex 3	- Wong, S.S. (1991), Chemistry of protein conjugation and cross-linking, CRC Press;
Annex 4	- Thorpe *et al* (1987), Cancer Res, 1987, 47, 5924-31. This paper describes the use of SMBT (sodium S-4-succinimidyloxycarbonyl-alpha-methyl benzyl thiosulfate) and SMPT (4-succinimidyloxycarbonyl-alpha-methyl-alpha(2-pyridyldithio)toluene); and
Annex 5	- Peeters *et al* (1989), J Immunol Methods. 1989, 120, 133-43. This paper describe the use of 4 coupling reagents, MHS (succinimidyl 6-(N-maleimido)-n-hexanoate), SMCC (succinimidyl 4-(N-maleimidomethyl)-cyclohexane-1-carboxylate), MBS (succinimidyl m-maleimidobenzoate), and SPDP.

Whilst it is true that the use of different coupling agents may have different effects (ie. improved, neutral, or adverse) on the biological activity of an agent according to US 09/529,130, I can confirm that the use of such coupling agents is (and was prior to October 1997) a matter of routine to a person of skill in the art. Thus, in principle, any member of the well known class of chemical coupling agent may be employed to couple a lectin to a polypeptide comprising a clostridial protease domain and a clostridial translocation domain. Moreover, having selected a particular coupling agent, it is (and was prior to October 1997) a matter of routine to confirm that the resulting agent has the requisite biological activity, as explained in more detail below.

Confirmation of L-chain function after chemical coupling may be tested by assaying for protease activity inherent to the L-chain.

By way of example, any one of the following three routine tests may be employed.

SNAP-25 (or synaptobrevin, or syntaxin) may be challenged with an "agent" to be tested, and then analysed by SDS-PAGE peptide separation techniques. Subsequent detection of peptides (eg. by silver staining) having molecular weights corresponding to the cleaved products of SNAP-25 (or other component of the neurosecretory machinery) would confirm the presence of a functional L-chain.

As an alternative, the "agent" may be tested by either *in vitro* challenge (see Examples 4-7 of US 09/529,130) or *in vivo* challenge in a mouse experiment (see Examples 8-9 of US 09/529,130).

As a further alternative, the "agent" may be tested by assaying for SNAP-25 (or synaptobrevin, or syntaxin) cleavage products via antibody-specific binding (see WO95/33850). In more detail, a specific antibody is employed for detecting cleavage of SNAP-25. Since the antibody recognises cleaved SNAP-25, but not uncleaved SNAP-25, identification of the cleaved product by the antibody confirms the presence of L-chain proteolytic function. By way of exemplification, such a method is described in Examples 2 and 3 of WO96/33273.

Confirmation of H-chain function after chemical coupling may be tested by assaying for translocation activity inherent to the H-chain.

Suitable methods are, for example, described by Shone *et al.* (1987) Eur. J. Biochem. 167, pp.175-180, and by Blaustein *et al.* (1987) FEBS 226 (1), pp.115-120. Copies of these documents are provided as Annex 6 and Annex 7.

The Shone *et al.* method employs artificial liposomes loaded with potassium phosphate buffer (pH 7.2) and radiolabelled NAD. Release of K^+ and NAD from the liposomes correlates with a positive result for channel forming activity and hence translocation activity. In this regard, K^+ release from liposomes may be measured using an electrode and NAD release calculated by measuring the radioactivity in the supernatant (see page 176, column 1, line 33 - column 2, line 17).

The Blaustein *et al.* method employs planar phospholipid bilayer membranes, which are used to test for channel forming activity. In more detail, salt solutions on either side of the membrane are buffered at a different pH - on the *cis* side, pH 4.7 or 5.5 and on the *trans* side, pH 7.4. The "agent" to be tested is added to the *cis* side of the membrane and electrical measurements are made under voltage clamp conditions, in order to monitor the flow of current across the membrane (see paragraph 2.2, pages 116-118). The presence of an active translocation function is confirmed by a steady rate of channel turn-on (i.e. a positive result for channel formation) -see paragraph 3, page 118.

Confirmation of Targeting Moiety (TM) function after chemical coupling may be tested by assaying for galactose-binding function inherent to the TM.

Suitable methods include:-

a simple haemagglutination assay [see, for example, Iglesias, J.L. *et al* (1982) – Purification and properties of a D-galactose/ N-acetyl-D-galactosamine-specific lectin from *Erythrina cristagalli*. Eur. J. Biochem., 123, pp. 247-252 – see Annex 8; and Arango, R. et al (1992) – Expression of *Erythrina corallodendron* lectin in *Escherichia coli*. Eur. J. Biochem., Apr 15, 205, pp. 575-581 – see Annex 9]; or

a simple assay to test for the ability to bind to immobilised sugars (eg. galactose) - see Iglesias, J.L. *et al* (1982) – Purification and properties of a D-galactose/ N-acetyl-D-galactosamine-specific lectin from *Erythrina cristagalli*. Eur. J. Biochem., 123, pp. 247-252

Haemagglutinin assays are well known in the art. In this regard, erythrocytes are known (and were so prior to 8 October 1997) to possess surface sugar (eg. galactose) residues, and are therefore susceptible to agglutination by galactose-binding lectins. Thus, the agglutinating activity of a galactose-binding lectin may be confirmed using heparinised human group O, Rh0 (D)+ erythrocytes. In more detail, the erythrocytes are prepared by trypsinisation, and 90 min incubation at room temperature. The resulting suspension is then challenged with an "agent" to be tested (diluted in PBS) and agglutination activity screened visually thereafter.

Immobilised sugar assays are well known in the art. In this regard, it is (and would have been prior to 8 October 1997) routine to test for active galactose-binding function. In more detail, a defined quantity of sugar [eg. N-acetyl galactosamine (GalNAc), or immobilised galactose (Gal)] is immobilised on to a matrix (eg. agarose). A "control" galactose-binding lectin mixture is added to the matrix and tumbled gently at room temperature (eg. for 30 minutes). The mixture is then transferred from the tumbling vessel to an empty 2.5ml polypropylene column and the eluant collected. After several column volume washes [eg. with 20 mM Hepes buffer pH 7.0], the buffer is switched to include 0.3 M GalNAc or Gal, with the result that specifically bound material is eluted. Using standard protein quantitation methodology (eg. BCA assay, Bradford assay, absorbance at 280nm), the ratio of unbound to specifically eluted material is readily estimated. This ratio may be used to determine the effect of coupling chemistry on the binding activity of the lectins.

Summary

I firmly believe that the specification of US 09/529,130 provides adequate written description and enablement details for a person of skill in the art to reproduce the invention across the scope of the pending claims (ie. Claims 63, 65, 66 and 71 as filed in response to the Official Action dated 9 July 2004).

I make this solemn declaration conscientiously believing the same to be true;

Signed ....Chaddock...........

Witnessed ....

Dated ....16th November 2004...........

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ANNEX 1

BIOGRAPHICAL SKETCH

Provide the following information for the key personnel in the order listed for Form Page 2.
Follow this format for each person. **DO NOT EXCEED FOUR PAGES.**

NAME	POSITION TITLE
Chaddock, John Andrew	Senior Scientist

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	YEAR(s)	FIELD OF STUDY
University of Durham, UK	BSc.	1988	Molecular Biology / Biochemistry
University of Warwick, UK	Ph.D.	1992	Protein Biochemistry
Open University, UK	Cert. Mgmt	2003	Management

NOTE: The Biographical Sketch may not exceed four pages. Items A and B (together) may not exceed two of the four-page limit. Follow the formats and instructions on the attached sample.

A. Positions and Honors. List in chronological order previous positions, concluding with your present position. List any honors. Include present membership on any Federal Government public advisory committee.

Positions and Employment

1992-1995	Postdoctoral Research, University of Warwick, UK
1995-1996	Postdoctoral Research, University of Warwick, UK
1996-2001	Scientist, Centre for Applied Microbiology & Research, Salisbury, UK
2002 – present	Senior Scientist, Health Protection Agency, Centre for Applied Microbiology & Research, Salisbury, UK

Other Experience

MSc Examiner in the field of protein toxin biochemistry
Co-supervisor of Ph.D. studentship at the University of Bath
Visiting lecturer at University of Bath

Professional Memberships

1988-present	Member of Biochemical Society, UK
2002-present	Member of International Association for the Study of Pain

B. Selected peer-reviewed publications (in chronological order).

1. Wales, R. Chaddock, J. A., Roberts, L. M. & Lord, J. M. (1992) Addition of an ER retention signal to the ricin A-chain increases the cytotoxicity of the holotoxin. *Exp. Cell Res.*, 203, 1-4.
2. Wales, R., Chaddock, J. A., Corben, E. B., Taylor, S. C., Roberts, L. M., Hartley, M. R. & Lord, J. M. (1993) Mutational analysis and possible applications of ribosome-inactivating proteins. In Beadle, D. J., Bishop, D. H. L., Copping, L. G., Dixon, G. K. & Hollomon, D. W. (eds.) BCPC Monograph No. 55: Opportunities for Molecular Biology in Crop Protection, pp99-111.
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In addition, co-inventorship on seven patents:

1. Purkiss, J. R., Chaddock, J. A., Quinn, C. P. & Foster, K. A. Inhibition of secretion from non-neuronal cells. WO 01/21213.
2. Chaddock, J. A., Alexander, F. C. G. & Foster K. A. Preparation of highly pure toxin fragments. WO 01/19863.
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5. Duggan, M. J. & Chaddock, J. A. Conjugates of Galactose-binding Lectins and Clostridial neurotoxins as Analgesics. WO 99/17806.
6. Shone, C. C., Quinn, C. P., Foster, K. A., Chaddock, J. A., Marks, P. R., Sutton, J. M., Stancombe, P. R. & Wayne, J. Recombinant Clostridium neurotoxin Fragments. Patent Application No. PCT/GB03/03824.
7. Foster, K.A. & Chaddock, J. A. Retargeted toxin fragments. UK application 0321344.4

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Bioconjugate Techniques

Greg T. Hermanson
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Rockford, Illinois

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Front cover illustration: A DNA double helix chemically modified at the N2 of a guanine residue to possess a γ-aminobutyric acid (GABA) group. The molecular model was kindly provided by Dr. George Pack of the University of Illinois College of Medicine at Rockford.

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Detailed Contents

Preface xxi
Acknowledgments xxv

PART I
Bioconjugate Chemistry

1. Functional Targets

- 1. Modification of Amino Acids, Peptides, and Proteins 3
  - 1.1. Protein Structure Reactivity 4
    - Amino Acids 4
    - Nucleophilic Reactions and the pI of Amino Acid Side Chains 12
    - Secondary, Tertiary, and Quaternary Structure 17
    - Prosthetic Groups, Cofactors, and Post-translational Modifications 19
    - Protecting the Native Conformation and Activity of Proteins 21
  - 1.2. Protein Cross-linking Methods 23
- 2. Modification of Sugars, Polysaccharides, and Glycoconjugates 27
  - 2.1. Carbohydrate Structure and Function 27
    - Basic Sugar Structure 27
    - Sugar Functional Groups 29
    - Polysaccharide and Glycoconjugate Structure 34
  - 2.2. Carbohydrate Cross-linking Methods 39
- 3. Modification of Nucleic Acids and Oligonucleotides 40
  - 3.1. Polynucleotide Structure and Function 41
    - Nucleotide Functional Groups 42
    - RNA and DNA Structure 53
  - 3.2. Polynucleotide Cross-linking Methods 55
- 4. Creating Specific Functional Groups 56
  - 4.1. Introduction of Sulfhydryl Residues (Thiolation) 56
    - Modification of Amines with 2-Iminothiolane (Traut's Reagent) 57
    - Modification of Amines with SATA 60
    - Modification of Amines with SATP 63
    - Modification of Amines with SPDP 64
    - Modification of Amines with SMPT 66
    - Modification of Amines with N-Acetylhomocysteinethiolactone 68
    - Modification of Amines with SAMSA 70

Modification of Aldehydes or Ketones with AMBH 71
Modification of Carboxylates or Phosphates with Cystamine 72
Use of Disulfide Reductants 76
Ellman's Assay for the Determination of Sulfhydryls 88
4.2. Introduction of Carboxylate Groups 90
Modification of Amines with Anhydrides 90
Modification of Sulfhydryls with Iodoacetate 98
Modification of Hydroxyls with Chloroacetic Acid 100
4.3. Introduction of Primary Amine Groups 100
Modification of Carboxylates with Diamines 101
Modification of Sulfhydryls with *N*-(β-Iodoethyl)trifluoroacetamide (Aminoethyl-8) 104
Modification of Sulfhydryls with Ethylenimine 105
Modification of Sulfhydryls with 2-Bromoethylamine 106
Modification of Carbohydrates with Diamines 107
Modification of Alkylphosphates with Diamines 109
Modification of Aldehydes with Ammonia or Diamines 109
Introduction of Arylamines on Phenolic Compounds 110
Amine Detection Reagents 112
4.4. Introduction of Aldehyde Residues 114
Periodate Oxidation of Glycols and Carbohydrates 114
Oxidase Modification of Sugar Residues 116
Modification of Amines with NHS-Aldehydes (SFB and SFPA) 117
Modification of Amines with Glutaraldehyde 118
4.5. Introduction of Hydrazide Functional Groups 120
Modification of Aldehydes with Bis-hydrazide Compounds 121
Modification of Carboxylates with Bis-hydrazide Compounds 123
Modification of Alkylphosphates with Bis-hydrazide Compounds 124
5. Blocking Specific Functional Groups 125
5.1. Blocking Amine Groups 126
Sulfo-NHS Acetate 127
Acetic Anhydride 127
Citraconic Anhydride 128
Maleic Anhydride 129
5.2. Blocking Sulfhydryl Groups 129
N-Ethylmaleimide 130
Iodoacetate Derivatives 131
Sodium Tetrathionate 131
Ellman's Reagent 132
Dipyridyl Disulfide Reagents 133
5.3. Blocking Aldehyde Groups 134
Reductive Amination with Tris or Ethanolamine 135
5.4. Blocking Carboxylate Groups 136
Tris or Ethanolamine plus EDC 136

2 The Chemistry of Reactive Groups

1. Amine-Reactive Chemical Reactions 137
1.1. Isothiocyanates 138
1.2. Isocyanates 138
1.3. Acyl Azides 139

71
mine 72
76
88
90
90
98
100
100
101
acetamide
104
105
106
107
109
109
110
112
114
114
116
A) 117
118
120
121
ls 123
unds 124
125
126
127
127
128
129
129
130
131
131
132
133
134
135
136
136
137
138
138
139

1.4. NHS Esters 139
1.5. Sulfonyl Chlorides 140
1.6. Aldehydes and Glyoxals 141
1.7. Epoxides and Oxiranes 142
1.8. Carbonates 142
1.9. Arylating Agents 143
1.10. Imidoesters 143
1.11. Carbodiimides 144
1.12. Anhydrides 145
2. Thiol-Reactive Chemical Reactions 146
2.1. Haloacetyl and Alkyl Halide Derivatives 147
2.2. Maleimides 148
2.3. Aziridines 148
2.4. Acryloyl Derivatives 149
2.5. Arylating Agents 149
2.6. Thiol–Disulfide Exchange Reagents 150
Pyridyl Disulfides 151
TNB-Thiol 151
Disulfide Reductants 152
3. Carboxylate-Reactive Chemical Reactions 152
3.1. Diazoalkanes and Diazoacetyl Compounds 153
3.2. Carbonyldiimidazole 153
3.3. Carbodiimides 154
4. Hydroxyl-Reactive Chemical Reactions 154
4.1. Epoxides and Oxiranes 155
4.2. Carbonyldiimidazole 155
4.3. *N,N'*-Disuccinimidyl carbonate or *N*-Hydroxysuccinimidyl chloroformate 156
4.4. Oxidation with Periodate 156
4.5. Enzymatic Oxidation 157
4.6. Alkyl Halogens 158
4.7. Isocyanates 158
5. Aldehyde- and Ketone-Reactive Chemical Reactions 159
5.1. Hydrazine Derivatives 159
5.2. Schiff Base Formation 159
5.3. Reductive Amination 160
5.4. Mannich Condensation 160
6. Active Hydrogen-Reactive Chemical Reactions 161
6.1. Diazonium Derivatives 161
6.2. Mannich Condensation 162
6.3. Iodination Reactions 162

7. Photoreactive Chemical Reactions 163
  7.1. Aryl Azides and Halogenated Aryl Azides 163
  7.2. Benzophenones 164
  7.3. Certain Diazo Compounds 164
  7.4. Diazirine Derivatives 165

PART II

Bioconjugate Reagents

3. Zero-Length Cross-linkers

1. Carbodiimides 169
  1.1. EDC 170
  1.2. EDC plus Sulfo-NHS 173
  1.3. CMC 177
  1.4. DCC 177
  1.5. DIC 180
2. Woodward's Reagent K 181
3. *N, N'*-Carbonyldiimidazole 183
4. Schiff Base Formation and Reductive Amination 185

4. Homobifunctional Cross-linkers

1. Homobifunctional NHS Esters 188
  1.1. DSP and DTSSP 192
  1.2. DSS and BS^3 194
  1.3. DST and Sulfo-DST 196
  1.4. BSOCOES and Sulfo-BSOCOES 198
  1.5. EGS and Sulfo-EGS 199
  1.6. DSG 201
  1.7. DSC 202
2. Homobifunctional Imidoesters 203
  2.1. DMA 204
  2.2. DMP 205
  2.3. DMS 206
  2.4. DTBP 207
3. Homobifunctional Sulfhydryl-Reactive Cross-linkers 208
  3.1. DPDPB 209
  3.2. BMH 210
4. Difluorobenzene Derivatives 211
  4.1. DFDNB 212
  4.2. DFDNPS 213

163
163
164
164
165

169
170
173
177
177
180
181
183
185

188
192
194
196
198
199
201
202
203
204
205
206
207
208
209
210
211
212
213

---

5. Homobifunctional Photoreactive Cross-linkers 214
    5.1. BASED 214
6. Homobifunctional Aldehydes 214
    6.1. Formaldehyde 215
    6.2. Glutaraldehyde 218
7. Bis-epoxides 220
    7.1. 1,4-Butanediol Diglycidyl Ether 221
8. Homobifunctional Hydrazides 222
    8.1. Adipic Acid Dihydrazide 222
    8.2. Carbohydrazide 223
9. Bis-diazonium Derivatives 224
    9.1. o-Tolidine, Diazotized 224
    9.2. Bis-diazotized Benzidine 225
10. Bis-alkylhalides 226

5. Heterobifunctional Cross-linkers

1. Amine-Reactive and Sulfhydryl-Reactive Cross-linkers 229
    1.1. SPDP, LC-SPDP, and Sulfo-LC-SPDP 230
    1.2. SMPT and Sulfo-LC-SMPT 232
    1.3. SMCC and Sulfo-SMCC 235
    1.4. MBS and Sulfo-MBS 237
    1.5. SIAB and Sulfo-SIAB 239
    1.6. SMPB and Sulfo-SMPB 242
    1.7. GMBS and Sulfo-GMBS 243
    1.8. SIAX and SIAXX 245
    1.9. SIAC and SIACX 247
    1.10. NPIA 247
2. Carbonyl-Reactive and Sulfhydryl-Reactive Cross-linkers 248
    2.1. MPBH 249
    2.2. M_2C_2H 250
    2.3. PDPH 252
3. Amine-Reactive and Photoreactive Cross-linkers 252
    3.1. NHS-ASA, Sulfo-NHS-ASA, and Sulfo-NHS-LC-ASA 255
    3.2. SASD 256
    3.3. HSAB and Sulfo-HSAB 260
    3.4. SANPAH and Sulfo-SANPAH 261
    3.5. ANB-NOS 263
    3.6. SAND 263
    3.7. SADP and Sulfo-SADP 265

3.8. Sulfo-SAPB 266
3.9. SAED 268
3.10. Sulfo-SAMCA 270
3.11. *p*-Nitrophenyl Diazopyruvate 272
3.12. PNP-DTP 274
4. Sulfhydryl-Reactive and Photoreactive Cross-linkers 275
4.1. ASIB 276
4.2. APDP 277
4.3. Benzophenone-4-iodoacetamide 279
4.4. Benzophenone-4-maleimide 280
5. Carbonyl-Reactive and Photoreactive Cross-linkers 282
5.1 ABH 282
6. Carboxylate-Reactive and Photoreactive Cross-linkers 283
6.1 ASBA 284
7. Arginine-Reactive and Photoreactive Cross-linkers 284
7.1 APG 285

6. Trifunctional Cross-linkers

1. 4-Azido-2-nitrophenylbiocytin-4-nitrophenyl ester 287
2. Sulfo-SBED 289

7. Cleavable Reagent Systems

1. Cleavage of Disulfides by Reduction 293
2. Periodate-Cleavable Glycols 294
3. Dithionite-Cleavable Diazo Bonds 295
4. Hydroxylamine-Cleavable Esters 295
5. Base Labile Sulfones 296

8. Tags and Probes

1. Fluorescent Labels 298
1.1. Fluorescein Derivatives 302
Amine-Reactive Fluorescein Derivatives 303
Sulfhydryl-Reactive Fluorescein Derivatives 307
Aldehyde/Ketone- and Cytosine-Reactive Fluorescein Derivatives 312
1.2. Rhodamine Derivatives 316
Amine-Reactive Rhodamine Derivatives 317
Sulfhydryl-Reactive Rhodamine Derivatives 326
Aldehyde/Ketone- and Cytosine-Reactive Rhodamine Derivatives 328

266
268
270
272
274
275
276
277
279
280
282
282
283
284
284
285
287
289
293
294
295
295
296
298
302
303
307
312
316
317
326
328

1.3. Coumarin Derivatives 331
  Amine-Reactive Coumarin Derivatives 332
  Sulfhydryl-Reactive Coumarin Derivatives 335
  Aldehyde- and Ketone-Reactive Coumarin Derivatives 339
1.4. BODIPY Derivatives 341
  Amine-Reactive BODIPY Derivatives 342
  Aldehyde/Ketone-Reactive BODIPY Derivatives 346
  Sulfhydryl-Reactive BODIPY Derivatives 350
1.5. Cascade Blue Derivatives 354
  Amine-Reactive: Cascade Blue Acetyl Azide 354
  Carboxylate-Reactive: Cascade Blue Cadaverine and Cascade Blue Ethylenediamine 355
  Aldehyde/Ketone-Reactive: Cascade Blue Hydrazide 357
1.6. Lucifer Yellow Derivatives 358
  Sulfhydryl-Reactive: Lucifer Yellow Iodoacetamide 359
  Aldehyde/Ketone-Reactive: Lucifer Yellow CH 361
1.7. Phycobiliprotein Derivatives 362
2. Bifunctional Chelating Agents and Radioimmunoconjugates 365
  2.1. DTPA 365
  2.2. DOTA, NOTA, and TETA 367
  2.3. DTTA 368
  2.4. DFA 369
  2.5. Use of Thiolation Reagents for Direct Labeling to Sulfhydryl Groups 370
3. Biotinylation Reagents 371
  3.1. Amine-Reactive Biotinylation Agents 373
    D-Biotin and Biocytin 373
    NHS-Biotin and Sulfo-NHS-Biotin 375
    NHS-LC-Biotin and Sulfo-NHS-LC-Biotin 377
    NHS-Iminobiotin 380
    Sulfo-NHS-SS-Biotin 382
  3.2. Sulfhydryl-Reactive Biotinylation Agents 384
    Biotin-BMCC 384
    Biotin-HPDP 386
    Iodoacetyl-LC-Biotin 388
  3.3 Carbonyl- or Carboxyl-Reactive Biotinylation Agents 390
    Biotin-Hydrazide and Biotin-LC-Hydrazide 390
    Biocytin Hydrazide 392
    5-(Biotinamido)pentylamine 394
  3.4. Photoreactive: Photobiotin 394
  3.5. Active-Hydrogen-Reactive: *p*-Aminobenzoyl Biocytin, Diazotized 398
4. Iodination Reagents 400
  4.1. Chloramine-T 403
  4.2. IODO-BEADS 405
  4.3. IODO-GEN 407
  4.4. Lactoperoxidase-Catalyzed Iodination 410

4.5. Iodinatable Modification and Cross-linking Agents 411
  Bolton–Hunter Reagent 412
  Iodinatable Bifunctional Cross-linking Agents 415

PART III
Bioconjugate Applications

9. Preparation of Hapten–Carrier Immunogen Conjugates

1. The Basis of Immunity 419
2. Types of Immunogen Carriers 421
  2.1. Protein Carriers 421
    KLH 422
    BSA and cBSA 423
    Thyroglobulin and OVA 426
    Tetanus and Diphtheria Toxoids 427
  2.2. Liposome Carriers 427
  2.3. Synthetic Carriers 428
3. Carbodiimide-Mediated Hapten–Carrier Conjugation 429
4. NHS Ester-Mediated Hapten–Carrier Conjugation 436
5. NHS Ester–Maleimide Heterobifunctional Cross-linker-Mediated Hapten–Carrier Conjugation 439
6. Active-Hydrogen-Mediated Hapten–Carrier Conjugation 446
  6.1. Diazonium Conjugation 446
  6.2. Mannich Condensation 449
7. Glutaraldehyde-Mediated Hapten–Carrier Conjugation 453
8. Reductive-Amination-Mediated Hapten–Carrier Conjugation 454

10. Antibody Modification and Conjugation

1. Preparation of Antibody–Enzyme Conjugates 460
  1.1. NHS Ester–Maleimide-Mediated Conjugation 461
    Activation of Enzymes with NHS Ester–Maleimide Cross-linkers 461
    Conjugation with Reduced Antibodies 463
    Conjugation with 2-Iminothiolane-Modified Antibodies 465
    Conjugation with SATA-Modified Antibodies 467
  1.2. Glutaraldehyde-Mediated Conjugation 470
  1.3. Reductive-Amination-Mediated Conjugation 472
    Activation of Enzymes with Sodium Periodate 474
    Activation of Antibodies with Sodium Periodate 475
    Conjugation of Periodate-Oxidized HRP to Antibodies by Reductive Amination 475
    Conjugation of Periodate-Oxidized Antibodies with Amine or Hydrazide Derivatives 476

gents 411
412
415
419
421
421
422
423
426
427
427
428
429
436
439
446
446
449
453
454
460
461
rs 461
463
465
467
470
472
474
475
475
476

1.4. Conjugation Using Antibody Fragments 478
Preparation of F(ab')$_2$ Fragments Using Pepsin 479
Preparation of Fab Fragments Using Papain 480
1.5. Removal of Unconjugated Enzyme from Antibody–Enzyme Conjugates 483
Immunoaffinity Chromatography 484
Nickel-Chelate Affinity Chromatography 486
2. Preparation of Labeled Antibodies 487
2.1. Fluorescently Labeled Antibodies 488
2.2. Radiolabeled Antibodies 490
2.3. Biotinylated Antibodies 492

11. Immunotoxin Conjugation Techniques

1. Properties and Use of Immunotoxin Conjugates 496
2. Preparation of Immunotoxin Conjugates 499
2.1. Preparation of Immunotoxin Conjugates via Disulfide Exchange Reactions 503
Pyridyldisulfide Reagents 503
Use of Cystamine, Ellman's Reagent, or S-Sulfonate 514
2.2. Preparation of Immunotoxin Conjugates via Amine- and Sulfhydryl-Reactive Heterobifunctional Cross-linkers 516
SIAB 517
SMCC 519
MBS 522
SMPB 524
2.3. Preparation of Immunotoxin Conjugates via Reductive Amination 524
Periodate Oxidation of Glycoproteins Followed by Reductive Conjugation 525
Periodate-Oxidized Dextran as Cross-linking Agent 525

12. Preparation of Liposome Conjugates and Derivatives

1. Properties and Use of Liposomes 528
1.1. Liposome Morphology 528
1.2. Preparation of Liposomes 531
1.3. Chemical Constituents of Liposomes 533
1.4. Functional Groups of Phospholipids 539
2. Derivatization and Activation of Lipid Components 540
2.1. Periodate Oxidation of Liposome Components 541
2.2. Activation of PE Residues with Heterobifunctional Cross-linkers 542
3. Use of Glycolipids and Lectins to Effect Specific Conjugations 548
4. Antigen or Hapten Conjugation to Liposomes 549
5. Preparation of Antibody–Liposome Conjugates 552

6. Preparation of Biotinylated or Avidin-Conjugated Liposomes 553
7. Conjugation of Proteins to Liposomes 556
    7.1. Coupling via the NHS Ester of Palmitic Acid 556
    7.2. Coupling via Biotinylated Phosphatidylethanolamine Lipid Derivatives 558
    7.3. Conjugation via Carbodiimide Coupling to Phosphatidylethanolamine Lipid Derivatives 559
    7.4. Conjugation via Glutaraldehyde Coupling to Phosphatidylethanolamine Lipid Derivatives 560
    7.5. Conjugation via DMS Cross-linking to Phosphatidylethanolamine Lipid Derivatives 562
    7.6. Coupling via Periodate Oxidation Followed by Reductive Amination 562
    7.7. Coupling via SPDP-Modified Phosphatidylethanolamine Lipid Derivatives 564
    7.8. Coupling via SMPB-Modified Phosphatidylethanolamine Lipid Derivatives 565
    7.9. Coupling via SMCC-Modified Phosphatidylethanolamine Lipid Derivatives 566
    7.10. Coupling via Iodoacetate-Modified Phosphatidylethanolamine Lipid Derivatives 567

13. Avidin–Biotin Systems

1. The Avidin–Biotin Interaction 570
2. Use of Avidin–Biotin in Assay Systems 572
3. Preparation of Avidin or Streptavidin Conjugates 575
    3.1. NHS Ester–Maleimide-Mediated Conjugation Protocols 575
    3.2. Periodate Oxidation/Reductive Amination Conjugation Protocols 580
    3.3. Glutaraldehyde Conjugation Protocol 583
4. Preparation of Fluorescently Labeled Avidin or Streptavidin 584
    4.1. Modification with FITC 585
    4.2. Modification with Lissamine Rhodamine B Sulfonyl Chloride 586
    4.3. Modification with AMCA–NHS 587
    4.4. Conjugation with Phycobiliproteins 588
5. Preparation of Hydrazide-Activated Avidin or Streptavidin 589
6. Biotinylation Techniques 590
7. Determination of the Level of Biotinylation 591

553
556
id 556
anolamine
558
s 559
ives 560
sphatidyl-
562
562
564
565
566
tidyl-
567
570
572
575
575
580
583
n 584
585
586
587
588
589
590
591

14. Preparation of Colloidal-Gold-Labeled Proteins

1. Properties and Use of Gold Conjugates 593
2. Preparation of Mono-disperse Gold Suspensions for Protein Labeling 597
    2.1. Preparation of 2-nm Gold Particle Sols 597
    2.2. Preparation of 5-nm Gold Particle Sols 598
    2.3. Preparation of 12-nm Gold Particle Sols 598
    2.4. Preparation of 30-nm Gold Particle Sols 598
3. Preparation of Protein A–Gold Complexes 599
4. Preparation of Antibody–Gold Complexes 600
5. Preparation of Lectin–Gold Complexes 601
6. Preparation of Avidin–Gold or Streptavidin–Gold Complexes 602

15. Modification with Synthetic Polymers

1. Protein Modification with Activated Polyethylene Glycols 606
    1.1. Trichloro-*s*-triazine Activation and Coupling 607
    1.2. NHS Ester and NHS Carbonate Activation and Coupling 609
    1.3. Carbodiimide Coupling of Carboxylate–PEG Derivatives 614
    1.4. CDI Activation and Coupling 615
    1.5. Miscellaneous Coupling Reactions 617
2. Protein Modification with Activated Dextrans 618
    2.1. Polyaldehyde Activation and Coupling 620
    2.2. Carboxyl, Amine, and Hydrazide Derivatives 623
    2.3. Epoxy Activation and Coupling 626
    2.4. Sulfhydryl-Reactive Derivatives 628

16. Enzyme Modification and Conjugation

1. Properties of Common Enzymes 630
    1.1. Horseradish Peroxidase 630
    1.2. Alkaline Phosphatase 632
    1.3. β-Galactosidase 633
    1.4. Glucose Oxidase 634
2. Preparation of Activated Enzymes for Conjugation 635
    2.1. Glutaraldehyde-Activated Enzymes 635
    2.2. Periodate Oxidation Techniques 636
    2.3. SMCC-Activated Enzymes 636

2.4. Hydrazide-Activated Enzymes 637
2.5. SPDP-Activated Enzymes 637
3. Preparation of Biotinylated Enzymes 637

17. Nucleic Acid and Oligonucleotide Modification and Conjugation

1. Enzymatic Labeling of DNA 640
2. Chemical Modification of Nucleic Acids and Oligonucleotides 643
  2.1. Diamine or Bis-hydrazide Modification of DNA 644
    Via Bisulfite Activation of Cytosine 645
    Via Bromine Activation of Thymine, Guanine, and Cytosine 647
    Via Carbodiimide Reaction with 5' Phosphates of DNA (Phosphoramidate Formation) 649
  2.2. Sulfhydryl Modification of DNA 651
    Cystamine Modification of 5' Phosphate Groups Using EDC 651
    SPDP Modification of Amines on Nucleotides 652
    SATA Modification of Amines on Nucleotides 654
  2.3. Biotin Labeling of DNA 656
    Biotin–LC-dUTP 656
    Photo-Biotin Modification of DNA 657
    Reaction of NHS-LC–Biotin with Diamine-Modified DNA Probes 658
    Biotin–Diazonium Modification of DNA 658
    Reaction of Biotin–BMCC with Sulfhydryl-Modified DNA 660
    Biotin–Hydrazide Modification of Bisulfite-Activated Cytosine Groups 661
  2.4. Enzyme Conjugation to DNA 662
    Alkaline Phosphatase Conjugation to Cystamine-Modified DNA Using Amine- and Sulfhydryl-Reactive Heterobifunctional Cross-linkers 663
    Alkaline Phosphatase Conjugation to Diamine-Modified DNA Using DSS 664
    Enzyme Conjugation to Diamine-Modified DNA Using PDITC 666
    Conjugation of SFB-Modified Alkaline Phosphatase to Bis-hydrazide-Modified Oligonucleotides 668
  2.5. Fluorescent Labeling of DNA 668
    Conjugation of Amine-Reactive Fluorescent Probes to Diamine-Modified DNA 670
    Conjugation of Sulfhydryl-Reactive Fluorescent Probes to Sulfhydryl-Modified DNA 671

References 673
Suppliers of Reagents and Devices for Bioconjugate Applications 727
Index 729

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ANNEX 4

New Coupling Agents for the Synthesis of Immunotoxins Containing a Hindered Disulfide Bond with Improved Stability *in Vivo*

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ABSTRACT

Two new coupling agents were synthesized for making immunotoxins containing disulfide bonds with improved stability *in vivo*: sodium *S*-4-succinimidyloxycarbonyl-α-methyl benzyl thiosulfate (SMBT) and 4-succinimidyloxycarbonyl-α-methyl-α(2-pyridyldithio)toluene (SMPT). Both reagents generate the same hindered disulfide linkage in which a methyl group and a benzene ring are attached to the carbon atom adjacent to the disulfide bond and protect it from attack by thiolate anions.

An immunotoxin consisting of monoclonal anti-Thy-1.1 antibody (OX7) linked by means of the SMPT reagent to chemically deglycosylated ricin A-chain had better stability *in vivo* than an immunotoxin prepared with 2-iminothiolane hydrochloride (2IT) which generates an unhindered disulfide linkage. About 48 h after i.v. injection into mice, one-half of the SMPT-linked immunotoxin present in the blood was in intact form and one-half as released free antibody, whereas equivalent breakdown of the 2IT-linked immunotoxin was seen at about 8 h after injection. Consequently, the blood levels of the SMPT-linked immunotoxin remained higher than those of the 2IT-linked immunotoxin despite loss of immunotoxin from the blood by other mechanisms. Forty-eight h after injection, 10% of the injected dose of the SMPT-linked immunotoxin remained in the bloodstream as compared with only 1.5% of the 2IT-linked immunotoxin.

The ability of immunotoxins prepared with the new reagents to inhibit protein synthesis by Thy-1.1-expressing AKR-A/2 lymphoma cells *in vitro* was identical to that of immunotoxins prepared with 2IT or *N*-succinimidyl-3-(2-pyridyldithio)propionate (SPDP). Clonogenic assays showed that fewer than 0.01% of AKR-A/2 cells survived exposure to high concentrations of OX7-abrin A-chain immunotoxins prepared with SMBT, 2IT, or SPDP. Twelve clones of cells which had survived treatment with the SMBT-linked immunotoxin were isolated. None of the clones was selectively resistant to the SMBT-linked immunotoxin when retested in cytotoxicity assays.

In conclusion, immunotoxins prepared with the new coupling agents should have improved antitumor activity *in vivo* because they are longer lived and do not break down so readily to release free antibody which could compete for the target antigens.

INTRODUCTION

Novel antitumor agents called "immunotoxins" have been synthesized in several laboratories by covalently linking the A-chain of ricin and other toxins to antibodies against tumor-associated antigens (reviewed in Refs. 1–5). These reagents bind to antigens on the target cell surface, are endocytosed, and the A-chain then traverses the membrane, probably of the endocytic vesicle, and kills the cell by inactivating its ribosomes.

Conjugation of the antibody and A-chain is generally accomplished by means of cross-linking agents that introduce a disulfide bond between the two proteins. Immunotoxins prepared with nonreducible linkages are consistently less cytotoxic than their disulfide-bonded counterparts indicating that reductive cleavage of the disulfide bond to release the A-chain in the cytosol may be an important step in the cytotoxic process (6, 7). The two disulfide coupling agents used in most laboratories are SPDP[2] (8) and 2IT (9). These agents are simple to use and give consistent products that are stable in *in vitro* systems and long-term storage.

The disulfide bond formed by the SPDP and 2IT reagents appears to be unstable *in vivo*. We have shown that, after injection into mice, immunotoxins prepared with these reagents break down with a half-life of about 8 h to release free antibody. This is true of immunotoxins prepared from the A-chains of abrin (10), native ricin (11) and ricin which has been chemically deglycosylated to prevent its clearance by the carbohydrate-recognition systems of the liver (11). In other laboratories, evidence has been found which supports (12–14) or opposes lability (15, 16). In those studies in which lability was not observed, the immunotoxins were prepared from native ricin A-chain and were cleared from the bloodstream so rapidly that the slower event of immunotoxin breakdown may not have been evident.

Breakdown of the linkage in the immunotoxin is a problem for two reasons: (*a*) there is less intact immunotoxin available to locate and kill the tumor cells; (*b*) the released antibody can compete with the immunotoxin for the target antigens (17) and, being much longer-lived (11), has greater opportunity to bind to them. The effectiveness of further injections of immunotoxin could therefore be diminished because the tumor cell antigens are masked by the released antibody from the first immunotoxin injection.

In the present study we synthesized two new coupling agents, SMBT and SMPT. These reagents were then used to prepare immunotoxins containing the same hindered disulfide linkage in which a methyl group and a benzene ring protect the disulfide bond from attack by thiolate anions. Immunotoxins prepared with the new reagents have improved *in vivo* stability and their toxicity to target cells is practically identical to that of immunotoxins prepared with SPDP or 2IT. A similar coupling agent, SPDB, with greater resistance to reduction was recently described by Worrell *et al.* (18). *In vivo* stability and cytotoxicity data for SPDB-linked immunotoxins have not yet been reported.

MATERIALS AND METHODS

Materials

Seeds of *Abrus precatorius* were kindly provided by Dr. S. Olsnes (Norsk Hydro's Institute for Cancer Research, Oslo, Norway). The seeds were of Indian origin. Crushed castor beans (*Ricinus communis*) were a gift from Croda Premier Oils, Ltd., Hull, England. The beans were from Central Africa.

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The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] To whom requests for reprints should be addressed.

[2] The abbreviations used are: SPDP, *N*-succinimidyl-3-(2-pyridyldithio)-propionate; SMBT, sodium *S*-4-succinimidyloxycarbonyl-α-methyl benzyl thiosulfate; SMPT, 4-succinimidyloxycarbonyl-α-methyl-α(2-pyridyldithio)toluene; SBT, sodium *S*-4-succinimidyloxycarbonyl benzyl thiosulfate; 2IT, 2-iminothiolane hydrochloride; SPDB, *N*-succinimidyl-3-(2 pyridyldithio)butyrate; DTT, dithiothreitol; dg.ricA, deglycosylated ricin A-chain; abrA, abrin A-chain; IC_{50}, concentration that reduced [^{3}H]leucine incorporation by 50%; SDS, sodium dodecyl sulfate; OX7, monoclonal antibody directed against Thy-1.1; R10, monoclonal antibody directed against human glycophorin; GSH, reduced glutathione.

The hybridoma cell line, MRC OX7, secreting a mouse IgG1 subclass antibody to the Thy-1.1 antigen, was kindly provided by Dr. A. F. Williams (MRC Cellular Immunology Unit, University of Oxford). Details of its derivation have been published by Mason and Williams (19). The hybridoma cell line, LICR-LON-R10, secreting a mouse IgG1 subclass antibody to human glycophorin was kindly supplied by Dr. P. A. W. Edwards (Ludwig Institute, Sutton, England).

The Thy-1.1-expressing AKR-A lymphoma cell line was obtained from Professor I. MacLennan (Department of Experimental Pathology, Birmingham University, Birmingham, England). It was recloned to remove a mutant subpopulation which was resistant to immunotoxins prepared using the SPDP reagent but sensitive to immunotoxins prepared using the 2IT reagent (10). The recloned line is designated AKR-A/2.

Tissue culture medium RPMI 1640 and fetal calf serum were purchased from Gibco-Biocult, Ltd. (Paisley, Scotland). Agarose (Sea Plaque) was from FMC Corporation (Rockland, ME). Microplates with 96 flat-bottomed wells and tissue culture plates with 24 flat-bottomed wells were purchased from Flow Laboratories (Irvine, Scotland).

Sodium [^{125}I]iodide (IMS 30) and L-[4,5-^{3}H]leucine (TRK 170) were obtained from Amersham International (Amersham, England). The Iodo-Gen reagent for protein iodination was from Pierce (United Kingdom) Ltd. (Chester, England).

Chromatography media were Sephacryl S-200, Sepharose 4B, Sephadex G25 (fine grade), and Blue Sepharose CL-6B from Pharmacia Ltd. (Milton Keynes, England).

SPDP was purchased from Pharmacia, Ltd., and 2IT from Sigma, Ltd. (Poole, England). Thin layer chromatography (SiO_2) plates were from Merck (Kieselgel 60F). All other reagents were of analytical grade.

Synthesis of SBT

Bromotoluic acid (5.29 g; 25 mmol) was suspended in dioxan (10 ml) and was mixed with a solution of sodium thiosulfate (6.45 g; 26 mmol) in water (6 ml). The mixture was stirred at 40°C for 3 h during which time the solid dissolved and the thiosulfate derivative of toluic acid then crystallized out. The crystals (m.p. approximately 255°C with decomposition) were washed with cold water and dried under vacuum at 45°C to constant weight (3.90 g; 14 mmol; 55%). The solid was dissolved in dry dimethylformamide (5 ml) and mixed with a solution of *N*-hydroxysuccinimide (1.82 g; 16 mmol) and dicyclohexylcarbodiimide (2.97 g; 14 mmol) each in dry dimethylformamide (5 ml). The mixture was stirred for 16 h at room temperature and the urea was removed by filtration. The solvent was removed from the filtrate by rotary evaporation at 40°C using an oil pump and the *N*-succinimidyl derivative was recrystallized from methanol/$CHCl_3$. The yield of the white crystals was 3.47 g (66%). The overall yield for the synthesis was 36%. Melting point determinations showed decomposition at 120°C. The analysis

Requires: C 37.14, H 3.36, N 3.62, S 16.60, Na 5.95
Found: C 36.63, H 2.78, N 3.33, S 16.27, Na 6.01

was consistent with the structure shown in Fig. 1 ($C_{13}H_4NO_8S_2Na$).

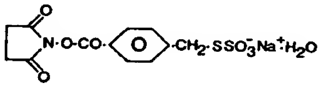

Fig. 1. The SBT reagent (formula weight, 385.4).

Synthesis of SMBT

p-Ethylbenzoic acid (5.15 g; 34 mmol) was dissolved in CH_2Cl_2 (45 ml) and solid *N*-bromosuccinimide (6.81 g; 38 mmol) was added followed by benzoylperoxide (0.08 g; 0.34 mmol) in CH_2Cl_2 (1 ml). The mixture was refluxed for 24 h. The white solid that remained was redissolved by the addition of further CH_2Cl_2 (40 ml) to the reaction mixture and the solution was extracted twice with water to remove succinimide. The CH_2Cl_2 solution was dried with anhydrous sodium sulfate and the solvent removed by rotary evaporation under reduced pressure. The white solid residue, α-bromoethylbenzoic acid, was recrystallized from isopropyl alcohol to give white crystals (m.p. 140°C) in good yield (5.62 g; 25 mmol; 72%). A solution of α-bromoethylbenzoic acid (0.60 g; 2.6 mmol) in dioxan (6 ml) was mixed with a solution of sodium thiosulfate (0.65 g; 2.6 mmol) in water (6 ml). The mixture was stirred for 16 h at room temperature and the solvents were removed in a vacuum at 40°C. The solid was washed with $CHCl_3$ and the thiosulfate derivative was recrystallized from water. The white crystals (shrinkage at 143°C; decomposition at 200°C) were recovered in poor yield (0.20 g; 0.72 mmol; 25%). The crystals were thoroughly dried and dissolved in dry dimethylformamide. The solution was mixed with solutions of dicyclohexylcarbodiimide (0.148 g; 0.72 mmol) and *N*-hydroxysuccinimide (0.090 g; 0.78 mmol) each in dry dimethylformamide (0.4 ml). The urea that had crystallized out after leaving the solution at room temperature for 16 h was removed by filtration. The solvent was removed from the filtrate by rotary evaporation at room temperature using an oil pump. The oily residue was redissolved in methyl ethyl ketone and undissolved solid was removed by filtration. The solvent was removed from the filtrate by rotary evaporation under reduced pressure and $CHCl_3$ was added to the residue which precipitated the product as a white solid that was dried under vacuum (0.17 g; 0.43 mmol; 72%; m.p. approximately 121°C with decomposition). The overall yield for the synthesis was 13%. The analysis

Requires: C 39.09, H 3.54, N 3.51, S 16.06, Na 5.76
Found: C 38.88, H 3.68, N 3.46, S 15.69, Na 5.68

was consistent with the structure shown in Fig. 2 ($C_{12}H_{12}NO_8S_2Na$).

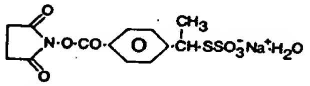

Fig. 2. The SMBT reagent (formula weight, 399.4).

Synthesis of SMPT

p-Ethylbenzoic acid was α-brominated and converted to the thiosulfate derivative as described in the preceding section. The thiosulfate (6.0 g; 20 mmol) was hydrolyzed by adding 5 N HCl (50 ml) and stirring at room temperature under nitrogen for 6 h. The reaction mixture was then extracted three times with ethyl acetate (50 ml) and dried over anhydrous sodium sulfate. The solvent was then removed in a vacuum to leave α-thioethylbenzoic acid (3.0 g; 16 mmol; 80%) as a white solid which was stored under nitrogen.

2-Pyridinesulfenylchloride was prepared by bubbling chlorine gas through a solution of 2,2-dipyridyldisulfide (2.3 g; 10 mmol) in dry dichloromethane (20 ml) for 30 min at room temperature. The solvent was then removed by rotary evaporation under reduced pressure and a solution of α-thioethylbenzoic acid (1.9 g; 10 mmol) in dry dioxan (10 ml) was added. The mixture was stirred vigorously overnight at room temperature under nitrogen. The yellow solid produced during the reaction was then partitioned between 0.05 M sodium phosphate buffer and ethyl acetate keeping the pH constant at 7.0. The organic layer was removed, dried over anhydrous sodium sulfate, and the solvent removed by rotary evaporation under reduced pressure to leave a yellow oil. Recrystallization from ethyl acetate/dichloromethane yielded colorless crystals (m.p. 130–132°C). The analysis

Requires: C 57.71, H 4.50, N 4.81, S 22.01
Found: C 57.92, H 4.63, N 4.89, S 21.99

was consistent with the product being 4-carboxy-α-methyl-α-(2-pyridyldithio)toluene ($C_{14}H_{13}NO_2S_2$).

To a solution of the 2-pyridyldithio derivative (1.9 g; 6.6 mmol) in dry dioxan (10 ml) were added dicyclohexylcarbodiimide (1.4 g; 6.8 mmol) and *N*-hydroxysuccinimide (0.79 g; 6.8 mmol) each dissolved in dry dioxan (approximately 4 ml). The mixture was stirred for 4 h at room temperature, filtered to remove the urea, and the solvent removed by rotary evaporation under reduced pressure. The product was purified by short column chromatography on Silica Gel H. Elution was effected with a gradient of CH_2Cl_2/ethyl acetate from 0 to 50% (v/v). On removal of the solvent, a colorless oil (0.6 g; 1.5 mmol; 24%) remained.

The product was homogeneous when analyzed by thin layer chromatography (SiO_2, ethyl acetate:CH_2Cl_2, 1:1) but attempts to crystallize it were unsuccessful. The overall yield for the synthesis was 2%. Nuclear magnetic resonance and infra-red analyses showed δH (d_4 methyl alcohol) 8.36 (1H, m, pyridyl); 7.96 (2H, m, phenyl); 7.73 to 7.53 (5H, m, phenyl and pyridyl); 7.06 (1H, m, pyridyl); 4.30 (1H, m, $C\underline{H}CH_3$); 2.96 (4H, S, *N*-hydroxysuccinimide ester); and 1.76 (3H, m, $\overline{C}\underline{H}CH_3$) ν_{max} (CH_2Cl_2) 2920, 1770, 1740, and 1605 cm^{-1}. These analyses were consistent with the structure shown in Fig. 3 ($C_{18}H_{16}N_2O_4S_2$).

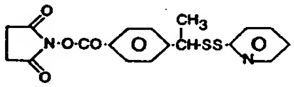

Fig. 3. The SMPT reagent (formula weight, 388.5).

Purification of Abrin A-Chain

Abrin was extracted from the seeds of *A. precatorius* by the method of Thorpe *et al.* (20). The toxin was split by reduction into its component chains and the A-chain was purified to homogeneity as described previously (21). The purified toxin and the A-chain (abrA) had median lethal dose values of 1.4 μg/kg and 12 mg/kg, respectively, when administered i.p. to adult BALB/c mice.

Deglycosylation and Purification of Ricin A-Chain

Ricin was purified from crushed castor beans by the method of Cumber *et al.* (21). A solution of the toxin (2.5 mg/ml) in 0.2 M sodium acetate buffer, pH 3.5, was treated for 1 h at 4°C with sodium metaperiodate and sodium cyanoborohydride at final concentrations of 10 and 20 mM respectively, as described by Thorpe *et al.* (22). This procedure results in the destruction of approximately 50% of the mannose and most of the fucose residues present on the A-chain. The *N*-acetylglucosamine and most of the xylose residues are unaffected (23). The deglycosylated ricin A-chain was separated from the B-chain and was extensively purified by the method of Fulton *et al.* (24). The dg.ricA had a median lethal dose value of 15 mg/kg (as compared with 30 mg/kg for native ricA) when administered i.p. to adult BALB/c mice.

Purification of Antibodies

The monoclonal antibodies OX7 and R10 were purified from the blood and ascitic fluid of hybridoma-bearing BALB/c mice by the method of Mason and Williams (19).

Preparation of Immunotoxins

Buffer Solutions. Two buffer solutions were used during the synthesis of the immunotoxins: (*a*) 0.05 M sodium borate, pH 9.0, containing 1.7% (w/v) NaCl ("borate buffer"); (*b*) 0.01 M Na_2HPO_4-0.0018 M KH_2PO_4-0.17 M NaCl-0.0034 M KCl-0.001 M EDTA, pH 7.5 ("phosphate-EDTA buffer").

Derivatization of Antibody with SMBT. To a solution of antibody (20 mg) in borate buffer (4 ml) was added SMBT (216 μl; 1 mg/ml) in dry dimethylformamide. The final concentrations of SMBT and antibody were 0.13 and 0.032 mM, giving a 4-fold M excess of SMBT over antibody. The solution was stirred for 1 h at room temperature and a solution of DTT (40 μl; 15.4 mg/ml) in borate buffer was added, giving a final DTT concentration of 1 mM. The solution was stirred gently for a further 1 h at room temperature and a solution of 5,5′-dithio-bis(2-nitrobenzoic acid) [Ellman's reagent (25)] (40 μl; 87.2 mg/ml) in dimethylformamide was added, giving a final concentration of Ellman's reagent of 2.2 mM. The mixture was stirred gently for 1 h at room temperature and was applied to a column (20 x 1.6 cm) of Sephadex G25 (fine) equilibrated in nitrogen-flushed phosphate-EDTA buffer. The protein that eluted in the void volume of the column was concentrated to 10 mg/ml in an Amicon ultrafiltration cell fitted with a YM2 membrane. The average number of activated disulfide groups introduced into each antibody molecule was determined by reducing a sample of derivatized antibody solution with DTT and measuring the absorption of the released 3-carboxylato-4-nitrothiophenolate ion which has a molar absorptivity of 1.36×10^4 M^{-1} cm^{-1} at 412 nm (25). The number of activated disulfide groups introduced using the above conditions ranged between 1.5 and 1.8/molecule of antibody.

Derivatization of Antibody with SMPT. To a solution of antibody (20 mg) in borate buffer (2.67 ml) was added SMPT (267 μl; 0.48 mg/ml) in dry dimethylformamide. The final concentrations of SMPT and antibody were 0.11 and 0.045 mM, giving a 2.4-fold M excess of SMPT over antibody. The dimethylformamide was used at 10% v/v to keep the SMPT soluble. The solution was stirred for 1 h at room temperature and was applied to a column (30 x 1.6 cm) of Sephadex G25 (fine) equilibrated in nitrogen-flushed phosphate-EDTA buffer. The protein that eluted in the void volume of the column was concentrated to 10 mg/ml in an Amicon ultrafiltration cell fitted with a YM2 membrane. The average number of α-methyl-α-(2-pyridyldithio)toluoyl groups introduced into each antibody molecule was determined by reducing a sample of derivatized antibody solution with DTT and measuring the absorption of the released pyridine-2-thione which has a molar absorptivity of 8.08×10^3 M^{-1} cm^{-1} at 343 nm (8). The number of α-methyl-α(2-pyridyldithio)toluoyl disulfide groups introduced using the above conditions ranged between 1.5 and 2.0/molecule of antibody.

Coupling of SMBT- and SMPT-derivatized Antibodies to A-chain. A solution of abrA (10 mg) or dg.ricA (10 mg) in phosphate-EDTA buffer (7 ml) was treated for 30 min at room temperature with 50 mM DTT and applied to a column (30 x 2.2 cm) of Sephadex G25 equilibrated in nitrogen-flushed phosphate-EDTA buffer. The A-chain fraction (about 35 ml) that eluted from the column was added directly to the concentrated antibody solution (10 mg/ml; 2 ml) in the Amicon ultrafiltration cell giving a molar excess of A-chain over antibody of 2.5-fold. The mixture was then concentrated to about 10 ml and incubated at room temperature for 72 h under nitrogen. The mixture was removed from the ultrafiltration cell and treated with 0.2 mM cysteine for 6 h at room temperature to inactivate any activated disulfide groups remaining in the antibody component of the immunotoxin. These conditions do not cause splitting of immunotoxin. If this step were omitted, 20 to 30% of the M_r 180,000 immunotoxin interacted with plasma constituents in both *in vivo* and *in vitro* experiments to form a covalent adduct mainly of M_r 240,000. It is possible that residual activated disulfide groups on the antibody component react with the thiol group of albumin (M_r 67,000) to form the adduct.

Preparation of SPDP- and 2IT-linked Immunotoxins. The SPDP and 2IT coupling agents were used to link abrA or dg.ricA to OX7 antibody. Full details of the procedures have been published previously for both SPDP (6, 21) and 2IT (10, 26).

Purification of the Immunotoxins. The products of the conjugation reactions above were applied to a column (90 x 2.2 cm) of Sephacryl S-200 equilibrated in 0.05 M sodium phosphate buffer, pH 7.5, and eluted with the same buffer solution. The fractions of immunotoxin that eluted with a molecular weight of approximately 180,000 were pooled and fractionated on a Blue Sepharose column to remove free antibody and immunotoxin molecules containing more than one molecule of A-chain as described previously (27).

Analysis by polyacrylamide gel electrophoresis in SDS showed that the immunotoxins had an apparent molecular weight of 180,000 and that they contained one molecule of antibody linked to one molecule of A-chain. The concentration of immunotoxin was determined from absorbance measurements at 280 nm. IgG-ricA (M_r 180,000) has an $E^{0.1\%}_{1cm}$ at 280 nm of 1.29 assuming values of 1.40 for the antibody and 0.765 for the A-chain (28). IgG-abrA (M_r 180,000) has an $E^{0.1\%}_{1cm}$ at 280 nm of 1.30 assuming values of 1.40 for the antibody and 0.787 for the A-chain (28).

The immunotoxins have the structures shown in Fig. 4. The antibody components of the OX7 immunotoxins fully retained antigen-binding activity, as judged by fluorescence-activated cell sorter analyses on AKR-A/2 cells treated with antibody or immunotoxin at saturating and subsaturating concentrations. The A-chain components fully retained their ability to inhibit protein synthesis in reticulocyte lysates (29).

Rate Constants

Bovine IgG in borate buffer (5 mg/ml) was treated with SBT, SMBT, SPDP, or 2IT to introduce an average of approximately 5 molecules of

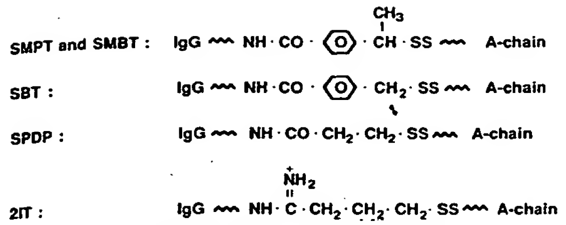
SMPT and SMBT :
IgG ~~ NH · CO · ⟨O⟩ · CH · SS ~~ A-chain
CH3
SBT :
IgG ~~ NH · CO · ⟨O⟩ · CH2 · SS ~~ A-chain
SPDP :
IgG ~~ NH · CO · CH2 · CH2 · SS ~~ A-chain
2IT :
IgG ~~ NH · C · CH2 · CH2 · CH2 · SS ~~ A-chain
NH2

Fig. 4. Linkages formed by the different coupling agents.

coupling agent per molecule of protein. The derivatized IgG was treated with 1 mM DTT for 1 h followed by 2.2 mM Ellman's reagent for 1 h. This generated the same activated disulfide-leaving group (*i.e.*, 3-carboxylato-4-nitrothiophenolate ion) in all the derivatives. The derivatized IgG preparations were desalted on columns (20 x 1.6 cm) of Sephadex G25 equilibrated in 0.025 M sodium phosphate, pH 7.4, containing 0.15 M NaCl.

The IgG derivatives were treated with DTT (0.01 to 0.1 mM) or glutathione (0.1 to 1 mM) at 25°C and the rate of release of 3-carboxylato-4-nitrothiophenolate ion was measured at 412 nm using a Shimadzu (Model UV 240) spectrophotometer. DTT or glutathione was added at the same time to the reference cell which contained underivatized bovine IgG which had been treated with DTT followed by Ellman's reagent in the same way as the IgG derivatives. The second order rate constants were calculated from the equation

$$k = \frac{2.303}{t(a - b)} \log \frac{b(a - x)}{a(b - x)}$$

where *a* is the initial molar concentration of activated disulfide groups in the derivatized protein solution, *b* is the initial molar concentration of DTT or glutathione, and *x* is the molar concentration of released 3-carboxylato-4-nitrothiophenolate ion at time *t* s after adding DTT or glutathione.

Toxicity to AKR-A Cells in Tissue Culture

[^{3}H]Leucine Incorporation Assays. A suspension of AKR-A/2 cells was prepared at 10^5 cells/ml in RPMI 1640 medium supplemented with 10% (v/v) heat-inactivated fetal calf serum, penicillin (200 units/ml), and streptomycin (100 μg/ml) ("complete medium"). The cells were incubated for 24 h at 37°C with immunotoxins and other test materials using the microplate method described previously (29). [^{3}H]Leucine (1 μCi) was then added to each culture (200 μl) and the radioactivity that the cells incorporated was measured 24 h later.

Clonogenic Assays. A suspension of AKR-A/2 cells was prepared at 2×10^5 cells/ml in complete medium. The suspension was distributed in 50-ml volumes into 300-cm^2 tissue culture flasks and complete medium or medium containing immunotoxin (1 ml) was added to give a final immunotoxin concentration of 1.3×10^{-8} M. The cells were incubated for 24 h at 37°C in an atmosphere of 5% CO_2 in humidified air. The cells were then washed three times with complete medium.

Cells which had been incubated in medium alone were suspended at 120, 240, 360, 480, and 600 cells/ml in complete medium. Cells which had been treated with immunotoxin were suspended at a range of concentrations between 10^4 and 10^7 cells/ml in complete medium. A solution of 0.24% w/v agarose in complete medium at 45°C was dispensed in 1-ml volumes into Petri dishes (35-mm diameter) and cooled for 1 h at 4°C to solidify the agarose. To aliquots of cell suspension (0.5 ml) in sterile tubes at 4°C was added a solution of 0.24% w/v agarose in complete medium (2.5 ml) at 45°C. The suspensions were then mixed, transferred in 1-ml volumes to the agar-coated Petri dishes, and were cooled for 1 h at 4°C to solidify the agarose. The Petri dishes were then incubated at 37°C for 10 days and the number of colonies containing about 100 cells or more was counted using an inverted microscope. The percentage of cells that survived exposure to the immunotoxins was calculated by comparing their cloning efficiency to that of untreated cells for that particular experiment. The cloning efficiency of untreated cells ranged from 65 to 76%.

Stability and Blood Clearance Measurements

Measurements of the stability and blood clearance rates of the immunotoxins were performed as previously described (11). Briefly, the purified OX7-SMPT-dg.ricA and OX7-2IT-dg.ricA immunotoxins were radioiodinated with ^{125}I to a specific activity of approximately 10^7 cpm/μg. Groups of three adult male specific-pathogen-free BALB/c/ICRF mice were given injections i.v. of 10 μg of radioiodinated immunotoxins and samples of blood were drawn from the tail vein at various time intervals and transferred to heparin-coated tubes. The radioactivity in the blood samples (50 μl) was measured. The samples were centrifuged at 10,000 × *g* for 2 min and the plasma was removed. The radioactivity in 20 μl plasma was counted and the samples were stored in liquid N_2. At the end of the experiment, volumes of plasma samples containing 8000 cpm each were electrophoresed on 5 to 10% polyacrylamide gels (1 mm thick) containing 1% SDS.

Autoradiographs of the dried gels were scanned and the area under the immunotoxin (M_r 180,000) peak and the released antibody (M_r 150,000) peak was divided by the total area under all the peaks to determine the proportion of radioactivity in the plasma that corresponded to intact immunotoxin or released antibody. Calibration experiments had previously shown that the area under each peak was directly proportional to the cpm it contained. Analysis of the immunotoxin by SDS-polyacrylamide gel electrophoresis under reducing conditions showed that the specific activity of the released antibody was somewhat less (9.1×10^6 cpm/μg) than that of the intact immunotoxin (10×10^6 cpm/μg). Correction was therefore made for this difference when calculating the amount of released antibody in the bloodstream. Clearance measurements were expressed as a percentage of the injected dose assuming that the mice had a blood volume of 2.18 ml/25 g body weight (30).

A two-compartment open pharmacokinetic model was fitted to the plasma levels of immunotoxins and released antibody using a computerized nonlinear least-squares regression analysis (31). A weighting function of $1/(Y + \hat{Y})^2$ was applied to all data points (32). These analyses yielded the half-lives of the immunotoxins in the α and β phases of clearance. Also, the half-lives of splitting of the immunotoxins to free antibody and A-chain were calculated using an extension of the same model to be described in a subsequent report.[3]

RESULTS

Rates of Reduction of IgG Derivatized with Various Coupling Agents

Bovine IgG was reacted with SMBT, SBT, SPDP, or 2IT and then treated with DTT followed by Ellman's reagent to form antibody derivatives in which the same activated disulfide group was present in all. The antibody derivatives differed only in the groups through which the activated disulfide group was attached to the protein, as in Fig. 4.

The release of 3-carboxylato-4-nitrothiophenolate ion when the antibody derivatives were treated with DTT or glutathione followed approximately second order kinetics, although the rate constant was greatest during the initial phase of reduction (Fig. 5). The activated disulfide groups reduced first probably occupied positions on the protein that were accessible to the reducing agents, whereas the more resistant groups were probably buried more deeply within the protein. The second order rate constants listed in Table 1 have been calculated at the point at which 50% of the leaving groups have been released and define the relative ease of reduction of the disulfide bonds formed by the different reagents.

The stability of the different linkages depended upon the degree of steric hindrance afforded by the groups adjacent to

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[3] D. C. Blakey, D. N. Skilleter, R. J. Price, H. Newell, and P. E. Thorpe, Comparison of the pharmacokinetics and hepatotoxic effects of saporin and ricin A-chain immunotoxins, manuscript in preparation.

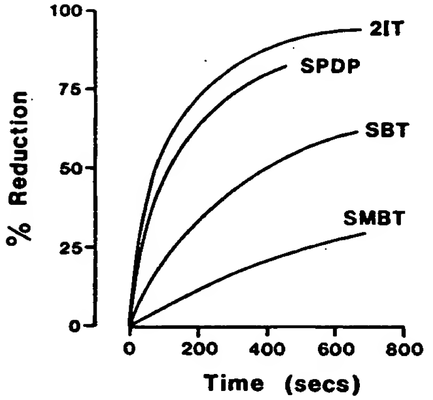

Fig. 5. Rates of reduction by DTT of IgG derivatized with various coupling agents. Bovine IgG was reacted with SMBT, SBT, 2IT, or SPDP and the derivatives were reduced with DTT and treated with Ellman's reagent to form antibody derivatives in which the same activated disulfide group was present in all. The derivatives were then treated with 0.03 mM DTT at pH 7.4 and 25°C. The rate of release of the 3-carboxylato-4-nitrothio phenolate group was followed spectrometrically at 412 nm. The A_{412} at various times after adding DTT is expressed as a percentage of the A_{412} after complete reduction with 5 mM DTT.

Table 1 *Rate constants for the reduction of IgG derivatized with various coupling agents by DTT or glutathione*

Bovine IgG was reacted with the above coupling agents and the derivatives were reduced with DTT and treated with an excess of Ellman's reagent to form antibody derivatives in which the same activated disulfide group was present in all. The derivatives were then treated with DTT (0.01 to 0.1 mM) or glutathione (0.1 to 1 mM) at pH 7.4 and 25°C. The rate of release of the 3-carboxylato-4-nitrothiophenolate group was measured spectrophotometrically as in Fig. 5. The second order rate constants shown below were calculated at the point at which 50% of the activated disulfide groups had been removed (see "Materials and Methods"). Repeated determinations gave results that did not differ by more than 15% from those shown.

Coupling agent	Rate constant (liters·mol⁻¹·s⁻¹)	
	DTT	Glutathione
SMBT	14	2.5
SBT	72	Not done
SPDP	250	52
2IT	320	165

the disulfide bond. As shown in Fig. 5 and Table 1, the SMBT reagent gave the most stable linkage, probably because the disulfide bond was protected by the methyl group and, to a lesser extent, by the benzene ring. The next most stable linkage was given by SBT which has a benzene ring in the same position as in SMBT but which lacks the methyl group. The least stable linkages were given by the SPDP and 2IT reagents in which the disulfide bond is essentially unprotected. Thus, in summary, the ratios of the rate constants for the reduction of the SMBT, SBT, SPDP, and 2IT linkages with DTT were 0.04:0.23:0.78:1, respectively. The reciprocals of these ratios give the relative stability of the SMBT, SBT, SPDP, and 2IT linkages as 24:4.5:1.3:1, respectively. These differences were even more marked when glutathione was used as the reducing agent (see Table 1).

Cytotoxicity to AKR-A/2 Lymphoma Cells

[^{3}H]Leucine Incorporation Assays. OX7-SMPT-dg.ricA had identical ability to OX7-2IT-dg.ricA to reduce protein synthesis by Thy-1.1-expressing AKR-A/2 cells in tissue culture (Fig. 6). Both immunotoxins reduced the [^{3}H]leucine incorporated by the cells by 50% at a concentration (the IC_{50}) of 6×10^{-13} M. They were about 10-fold more potent even than ricin which had an IC_{50} of 8×10^{-12} M. The toxic effects were specific. Unconjugated OX7 was not toxic at 10^{-7} M and unconjugated dg.ricA and the control immunotoxin, R10-SMPT-dg.ricA, were only toxic at concentrations in excess of 10^{-8} M.

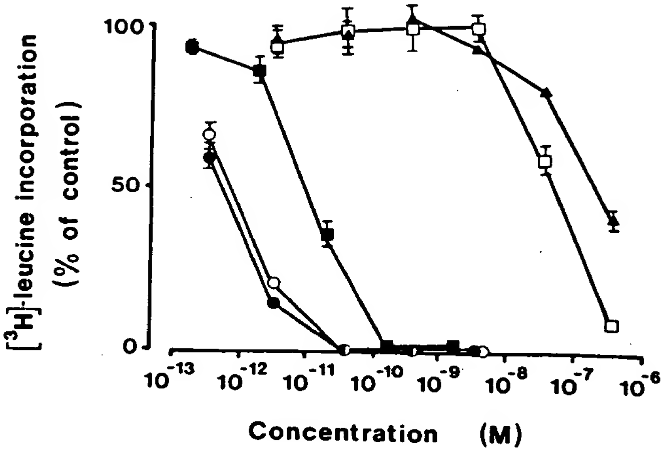

Fig. 6. Cytotoxic effects of OX7-SMPT-dg.ricA (○) and OX7-2IT-dg.ricA (●) upon AKR-A/2 lymphoma cells in tissue culture. The cells were incubated for 48 h with the immunotoxins or with R10-SMPT-dg.ricA (▲), dg.ricA (□), or ricin (■). *Points,* geometric means of triplicate measurements of [^{3}H]leucine incorporated by the cells during the final 24-h period of culture expressed as a percentage of the incorporation in untreated cultures. *Bars,* one SD about the mean unless smaller than the *points* as plotted. Mean [^{3}H]leucine incorporation in untreated cultures was 42,000 dpm.

Table 2 *Cytotoxic effects of OX7-abrA immunotoxins upon AKR-A/2 cells in vitro*

Immunotoxin	IC_{50} in [^{3}H]leucine uptake assays[a] (M)	Clonogenic assays[b] (% surviving cells)
OX7-SMBT-abrA	2.6×10^{-12}	0.0086 ± 0.0003
OX7-SPDP-abrA	2.0×10^{-12}	0.0049 ± 0.0021
OX7-2IT-abrA	3.0×10^{-12}	0.0037 ± 0.0002
OX7-abrA cocktail[c]	3.3×10^{-12}	0.0053 ± 0.0003
R10-SMBT-abrA	$>3 \times 10^{-8}$	90.0 ± 8.5
R10-SPDP-abrA	$>3 \times 10^{-8}$	73.5 ± 12.0
R10-2IT-abrA	$>3 \times 10^{-8}$	74.0 ± 1.4

[a] IC_{50} as determined in experiments such as is shown in Fig. 6.

[b] Immunotoxins were applied to the cells at 1.3×10^{-8} M. Results are the arithmetic means ± one SD of triplicate determinations. The plating efficiency of untreated cells was 76%.

[c] The cocktail contained SPDP-, 2IT- and SMBT-linked immunotoxin at 0.43×10^{-8} M each (total, 1.3×10^{-8} M).

In other experiments, OX7-abrA immunotoxins prepared with SMBT, SPDP, and 2IT were found to have very similar cytotoxicity to AKR-A/2 cells. Their IC_{50} values in [^{3}H]leucine incorporation assays ranged between 2.0 and 3.0×10^{-12} M (Table 2). Again, their toxic effects were specific. Control immunotoxins prepared from the R10 antibody were not toxic to AKR-A/2 cells at concentrations as high as 3×10^{-8} M, and none of the OX7-abrA immunotoxins was toxic to EL4 cells, a mouse lymphoma line which lacks Thy-1.1, at 3×10^{-8} M.

Clonogenic Assays

Clonogenic assays were used to quantify the survival of AKR-A/2 cells after treatment with the different OX7-abrA immunotoxins at the saturating concentration of 1.3×10^{-8} M. The 2IT- and SPDP-linked immunotoxins killed all but 0.0037 and 0.0049% of the cells, respectively, whereas the SMBT-linked immunotoxin killed all but 0.0086% of the cells (Table 2). This difference between the killing obtained with the SMBT-linked immunotoxin and that with the other two immunotoxins is statistically significant ($P < 0.05$) but is very small in view of the fact that more than 99.99% of the cells were killed with all three immunotoxins. A similar significant difference was obtained when the experiment was repeated.

Twelve clones of cells which survived exposure to OX7-SMBT-abrA were isolated and their sensitivity to the SMBT-, 2IT-, and SPDP-linked immunotoxins was tested in [^{3}H]leucine incorporation assays. Of 11 clones, one clone was fully resistant to all 3 immunotoxins, 4 were fully sensitive, and 6 showed intermediate sensitivity. None of these clones showed more than a 3-fold difference in their sensitivity to any individual immunotoxin indicating that the type of linkage used to form the immunotoxin does not usually affect the nature of the surviving cells (results not shown). In contrast, the 12th clone (S6A2) was 5- to 10-fold more sensitive to the 2IT-linked immunotoxin than it was to the other two immunotoxins. The IC_{50} values for this clone were 1×10^{-9} M with the 2IT-linked immunotoxin and 5×10^{-9} and 1×10^{-8} M with the SPDP- and SMPT-linked immunotoxins, respectively. Importantly, none of the clones studied was resistant only to the SMBT-linked immunotoxin suggesting that an inability to split the hindered disulfide bond in the SMBT linkage was not a cause of mutant cell survival.

Stability and Clearance Rate of Immunotoxins *in Vivo*

Fig. 7 shows autoradiographs of SDS gels of blood samples from mice given injections of radioiodinated OX7-SMPT-dg.ricA or OX7-2IT-dg.ricA at various earlier time intervals. The immunotoxin preparations that were injected contained a single major component (M_r 180,000) consisting of one molecule of antibody and one molecule of A-chain. After injection, both immunotoxins broke down to give a long-lived product (M_r 150,000) corresponding to free antibody. At later time points (Fig. 7, *lanes 9* and *10*) a minor component (M_r 210,000) was also seen on the gels. Free A-chain (M_r 30,000) was not seen at any time point probably because it was very rapidly cleared (33).

The rate at which OX7-SMPT-dg.ricA broke down to release free antibody *in vivo* was slower than that of OX7-2IT-dg.ricA. Plasma samples contained approximately equal amounts of intact immunotoxin and released antibody 48 h after injection of OX7-SMPT-dg.ricA (Fig. 7*a*, *lane 8*) as compared with after about 8 h in recipients of OX7-2IT-dg.ricA (Fig. 7*b*, *lane 6*). The released antibody had a specific activity of 9.1×10^6 cpm/µg which is lower than that of the intact immunotoxin (10×10^6 cpm/µg) so that the true amount of released antibody is slightly higher than appears to be present on the gels. The rate of splitting of OX7-2IT-dg.ricA was very similar to that of OX7-2IT-abrA and OX7-SPDP-abrA which, as we previously reported (10), have the same stability.

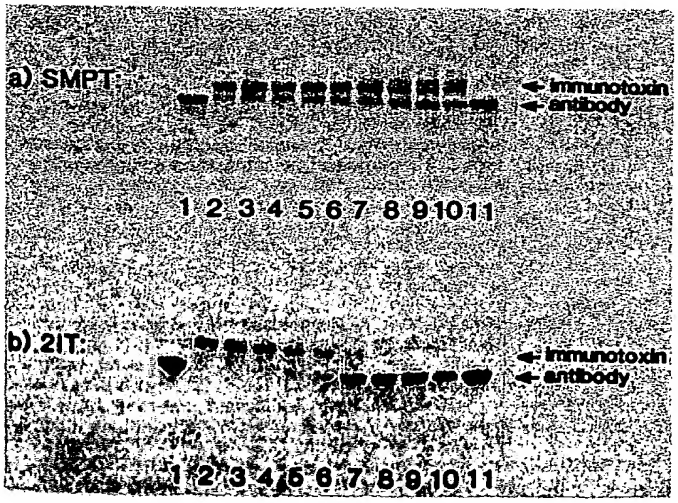

Fig. 7. Improved *in vivo* stability of OX7-dg.ricA prepared with SMPT. Autoradiographs of SDS-polyacrylamide (5 to 10%) gels of plasma samples taken at various time intervals from mice which had been given i.v. injections of (*a*) OX7-SMPT-dg.ricA or (*b*) OX7-2IT-dg.ricA. *a* and *b*, *lanes 1* and *11*, radioiodinated native OX7 antibody (M_r 150,000); *lane 2*, immunotoxin (mainly M_r 180,000) before injection. *a*, *lanes 3* to *10*, plasma samples taken 0.15, 2, 4, 8, 24, 48, 72, and 96 h after injection, respectively; *b*, *lanes 3* to *10*, plasma samples taken 0.15, 2, 4, 8, 24, 48, 72, and 120 h after injection, respectively.

The breakdown products of the immunotoxins were characterized by absorbing the plasma samples with anti-ricin antibody coupled to Sepharose and rerunning the gels. This procedure entirely removed the intact immunotoxin (M_r 180,000) leaving behind the M_r 150,000 component corresponding to released antibody and the M_r 210,000 component (results not shown). Absorption of the plasma samples with Sepharose coupled to an antibody of irrelevant specificity removed none of the radioiodinated components, confirming that the absorption of immunotoxin by antiricin coupled to Sepharose was antigen specific. The M_r 210,000 component therefore lacked ricin A-chain and probably arose by displacement of the A-chain from the immunotoxin by a serum component with a calculated molecular weight of approximately 60,000. One possibility is that this serum component is albumin (M_r 67,000) which, having a free thiol group, could potentially displace the A-chain and remain attached to the antibody.

In Fig. 8 are shown the amounts of OX7-SMPT-dg.ricA and OX7-2IT-dg.ricA remaining in intact (M_r 180,000) form in the blood plasma of mice at various times after injection. The clearance curves were biphasic, having an initial rapid α-phase followed by a slower β-phase. A computerized analysis of the clearance data using an open two-compartment pharmacokinetic model gave α-phase half-lives of 1.2 ± 0.2 (SE) h for OX7-SMPT-dg.ricA and 2.3 ± 0.3 h for OX7-2IT-dg.ricA. In the β-phase, the half-lives were 22 ± 1 h for OX7-SMPT-dg.ricA and 11 ± 1 h for OX7-2IT-dg.ricA. The α- and β-phase half-lives for native OX7 antibody were 6.7 ± 1.2 h and 118 h, respectively. Using a computerized analysis to be described elsewhere,[3] the OX7-SMPT-dg.ricA was calculated to split up to give free antibody with a half-life of 21.8 h as compared with 6.5 h for OX7-2IT-dg.ricA.

As a consequence of its better stability, 10% of the injected dose of OX7-SMPT-dg.ricA remained intact in the blood plasma 48 h after injection as compared with 1.5% of OX7-2IT-dg.ricA and 35% of native OX7 antibody (Fig. 8).

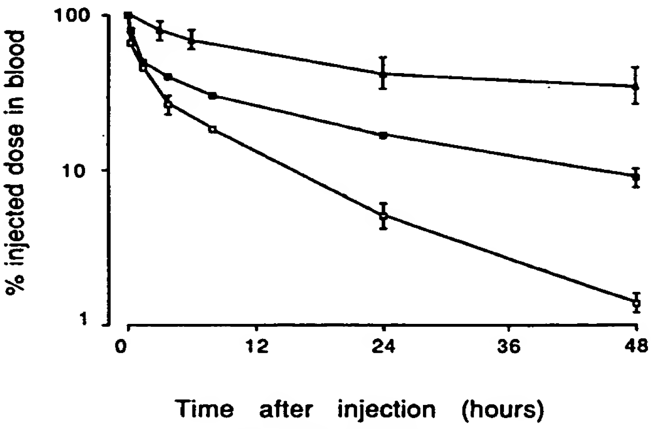

Fig. 8. Blood clearance rates of OX7-SMPT-dg.ricA (■), OX7-2IT-dg.ricA (□), and OX7 (Δ). Mice were given i.v. injections of radioiodinated immunotoxins and blood samples were removed at various time intervals later. The percentage of the injected dose that corresponded to intact (M_r 180,000) immunotoxin was determined by scanning autoradiographs of SDS gels such as those in Fig. 7. *Points*, geometric mean and SD (*bars*) of results obtained in three mice. OX7 derivatized with 2IT or SMPT followed by reduction and alkylation had identical blood clearance rates to native OX7 antibody (results not shown).

In contrast with the *in vivo* results, breakdown of the immunotoxins was not seen when they were incubated at 37°C for 24 h in 2 ml of mouse plasma or heparinized whole blood.

DISCUSSION

In the present study, we synthesized two new coupling agents, SMBT and SMPT, for preparing immunotoxins containing disulfide bonds with improved stability *in vivo.*

The SMBT and SMPT reagents generate the same protected disulfide linkage in which a methyl group and a benzene ring are attached to the carbon atom adjacent to the disulfide bond. However, the SMPT reagent is to be preferred for forming immunotoxins for two reasons: (*a*) it is simpler to use because it introduces the activated disulfide group in a single step, whereas the SMBT reagent introduces a thiosulfate group which has to be reduced and the resultant thiol activated with Ellman's reagent; (*b*) the SMPT coupling procedure does not involve exposing the antibody to DTT which potentially could cleave interchain disulfide bonds in the antibody and risk light chain loss.

In model experiments with antibody derivatized with different coupling agents, the protected disulfide bond formed by the SMBT reagent was found to have 24-fold greater resistance to reduction with DTT than the unprotected disulfide bonds formed by the SPDP and 2IT reagents. Both the benzene ring and the α-methyl group contribute to the greater stability of the SMBT linkage. This is indicated by our finding that antibody derivatized with a further coupling agent, SBT, which gives a linkage having a benzene ring in the same position as in the SMBT linkage but which lacks the α-methyl group, was about four times more resistant to thiol attack than the SPDP and 2IT linkages (see Table 1).

A similar coupling agent, SPDB, was recently synthesized by Worrell *et al.* (18). The SPDB reagent also generates a linkage with a methyl group substituted on the carbon atom adjacent to the disulfide bond but it has a smaller $—CH_2 \cdot CH_2—$ group in place of the benzene ring in the SMPT linkage and so may have inferior stability.

A dg.ricA immunotoxin prepared with SMPT broke down more slowly *in vivo* to release free antibody than an immunotoxin containing the unhindered 2IT linkage. Consequently, the β-phase half-life of the SMPT-linked immunotoxin was increased to 22 h as compared with 11 h for the immunotoxin prepared with the unhindered disulfide bond. In the study by Worrell *et al.* (18) increased stability of the SPDB linkage was not directly demonstrated but was inferred from the fact that the β-phase half-life of the SPDB-linked immunotoxin was increased to 14 h as compared with 10 h for an immunotoxin prepared with an unhindered linkage.

The mechanism by which immunotoxins are broken down *in vivo* is unknown. It has been suggested from the finding that immunotoxins do not break down significantly when incubated in mouse plasma or whole blood *in vitro*, that the splitting must occur within one of the organs or tissues of the animal, possibly the liver (11). However, this is not necessarily true, because GSH, the major free plasma thiol, is very rapidly lost from plasma *in vitro* by mechanisms that do not appear to involve oxidation by molecular oxygen (34). *In vivo*, GSH is continually being manufactured by the liver and is maintained in plasma at a level of about 24 μM. Thus it is possible that the disulfide bond in immunotoxins is slowly split by GSH in the blood *in vivo* and that placing hindering groups around the disulfide bond protects it from attack. Alternatively, the splitting could be due to the action of a disulfide reductase and the hindering groups frustrate enzymatic attack.

OX7-abrA and OX7-dg.ricA prepared with the SMBT or SMPT reagents had identical ability to inhibit [^{3}H]leucine incorporation by AKR-A/2 cells to immunotoxins prepared with SPDP or 2IT. We have since obtained similar results in other *in vitro* test systems using immunotoxins constructed from antibodies with a variety of different specificities. Thus strengthening the linkage in the immunotoxin does not weaken their cytotoxic activity as measured by gross reductions in protein synthesis 24 to 48 h after adding the immunotoxin to the cells.

Clonogenic assays revealed that 0.009% of AKR-A/2 cells survived exposure to high concentrations of OX7-SMBT-abrA whereas the survival after treatment with OX7-SPDP-abrA and OX7-2IT-abrA was 0.005 and 0.004%, respectively. This difference was statistically significant and prompted us to examine the immunotoxin sensitivity of 12 clones of cells that had survived exposure to the SMBT-linked immunotoxin. None of the clones was selectively resistant to the SMBT-linked immunotoxin when retested with the immunotoxins showing that an inability to split the hindered disulfide linkage and release the A-chain within the cytosol was not a cause of mutant cell survival, or, if it were, it was not a stable mutation.

In conclusion, immunotoxins prepared using the SMBT and SMPT reagents should have superior antitumor activity *in vivo* because, being more stable, they have more time to locate and kill the tumor cells and they release less free antibody which can compete for the target antigens.

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ANNEX 5

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Comparison of four bifunctional reagents for coupling peptides to proteins and the effect of the three moieties on the immunogenicity of the conjugates

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Peptide-carrier conjugates are widely used to raise antipeptide antibodies. In a model system using angiotensin and tetanus toxoid as the peptide and the carrier protein respectively, four cross-linking reagents were employed to study their effect on the immunogenicity of the conjugates. Optimization of the conjugation method for these heterobifunctional reagents, all succinimidyl active esters, resulted in well-defined conjugates of predictable composition. ELISA assays were performed to compare the antigenicity and the immunogenicity of the conjugates. The antipeptide antibody titres were of the order of 2×10^4–2×10^5. The anti-carrier antibody titres were high, in spite of the modification of the protein. Three of the four coupling reagents used for conjugation were of the 'maleimide' type: succinimidyl 6-(*N*-maleimido)-*n*-hexanoate (MHS), succinimidyl 4-(*N*-maleimidomethyl)-cyclohexane-1-carboxylate (SMCC) and succinimidyl *m*-maleimidobenzoate (MBS). One coupling reagent contained an activated disulphide: succinimidyl 3-(2-pyridyldithio)propionate (SPDP). The constrained linkers originating from SMCC and MBS induced very high *linker*-specific antibody levels. The more flexible non-aromatic linkers originating from MHS and SPDP showed almost no reactivity. For this reason and since the thioether linkage is more stable than the disulphide bond, we recommend MHS as the crosslinking reagent of choice.

Key words: Antipeptide antibody; Conjugation method; Cross-linking reagent; Linker-specific antibody; Peptide-protein conjugate

Introduction

Antipeptide antibodies have become important tools in many research fields; they have been used to identify new gene products, to analyse the functional domains of enzymes, for protein purification, for assaying proteins in immunochemical assays and to check the potential efficacy of synthetic peptide vaccines (for review see Moser et al., 1985; Walter, 1986).

In general, peptides consisting of 10–30 amino acid residues do not elicit antibodies following

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The *nomenclature* used is in accord with the rules and recommendations of the IUPAC-IUB Joint Commission on Biochemical Nomenclature: Eur. J. Biochem. 138, 9–37 (1984).

Abbreviations: Ata, acetylthioacetyl; MHS, succinimidyl 6-(*N*-maleimido)-*n*-hexanoate; Mh, 6-maleimidohexanoyl; SMCC, succinimidyl 4(*N*-maleimidomethyl)-cyclohexane-1-carboxylate; MBS, succinimidyl *m*-maleimidobenzoate; SATA, succinimidyl *S*-acetylthioacetate; SPDP, succinimidyl 3-(2-pyridyldithio)propionate; NHMe, methylamide; OPfp, pentafluorophenyloxy; TT, tetanus toxoid; DT, diphtheria toxoid; AI, [Val^5]-angiotensin I.

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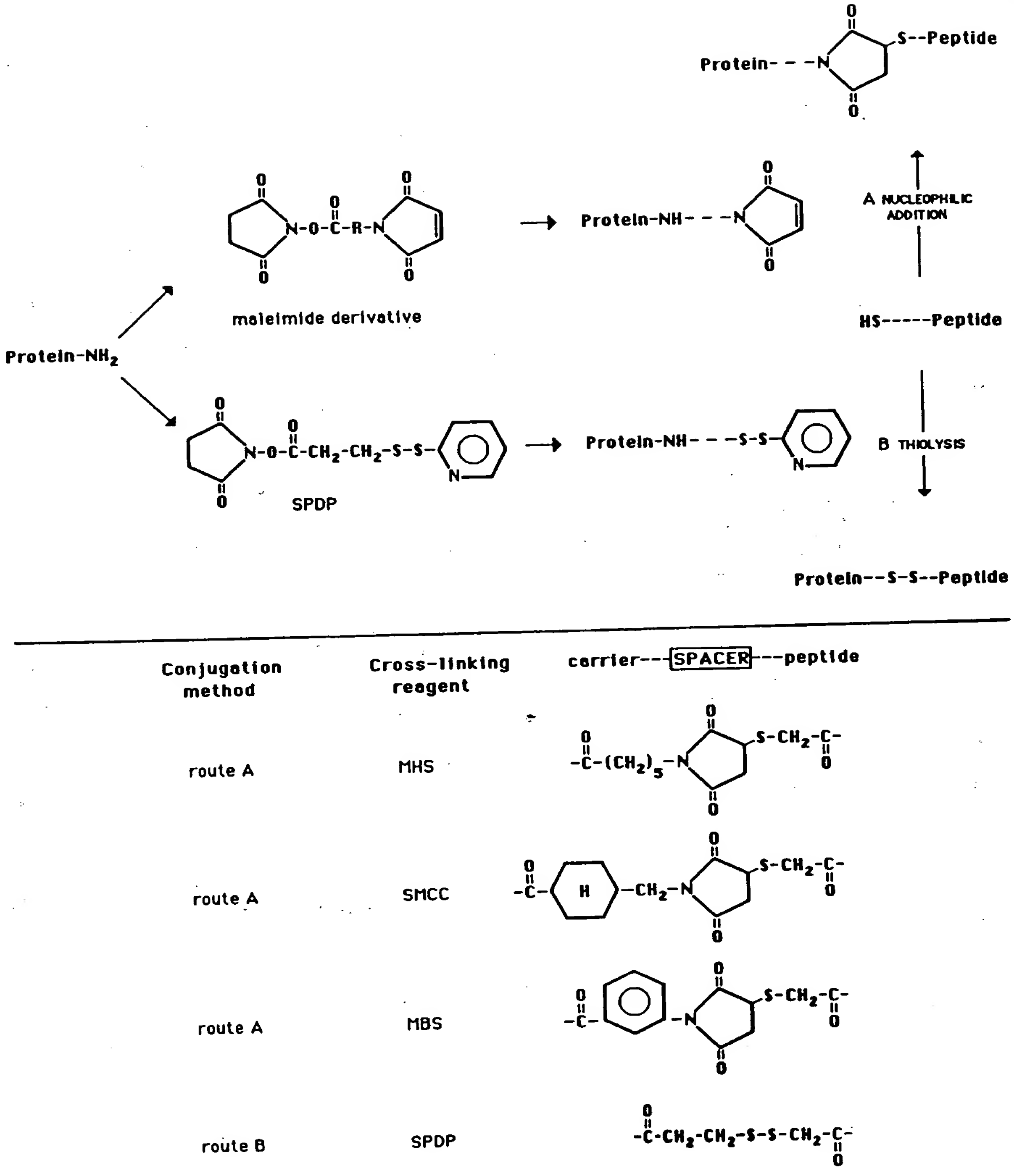
Protein- - -N
S--Peptide
A NUCLEOPHILIC ADDITION
Protein-NH₂
maleimide derivative
N-O-C-R-N
Protein-NH- - -N
HS-----Peptide
N-O-C-CH₂-CH₂-S-S-
SPDP
Protein-NH- - -S-S-
B THIOLYSIS
Protein--S-S--Peptide
Conjugation method
Cross-linking reagent
carrier---SPACER---peptide
route A
MHS
-C-(CH₂)₅-N
S-CH₂-C-
route A
SMCC
H
-CH₂-N
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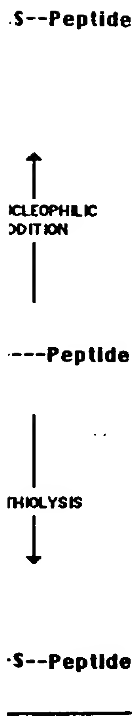
MBS
route B
SPDP
-C-CH₂-CH₂-S-S-CH₂-C-

Fig. 1. Synthesis of protein-peptide conjugates.



immunization. To induce immunogenicity, peptides are coupled to macromolecular carriers, usually proteins such as bovine serum albumin (BSA), keyhole limpet haemocyanin (KLH) and ovalbumin, to synthetic carriers such as multichain poly(DL-Ala)-(L-Lys) (Audibert, 1982) and polytuftsin (Trudelle, 1987) or they are incorporated into liposomes, micelles or immuno-stimulating complexes known as iscoms (Morein et al., 1984). In general, bifunctional reagents are required to couple peptides to proteins. Since peptides and proteins contain several functional groups, conjugation using carbodiimides (Goodfriend et al., 1964; Davis et al., 1984) or homobifunctional reagents (e.g., glutardialdehyde (Avrameas, 1969; Pfaff et al., 1982) and bis-diazotized benzidine (Walter et al., 1980; Tamura and Bauer, 1982)) generates a great number of different products ('chaos' coupling). In order to obtain the best-defined product heterobifunctional cross-linkers should be used in such a way that the peptide will be coupled specifically and in a predictable fashion to the carrier. Various aspects of chemical cross-linkers and the modification of proteins have been reviewed by Han et al. (1984) and Feeney (1987).

A useful method for preparing peptide-carrier conjugates involves taking advantage of the fast nucleophilic addition of a thiol group to the double bond of a maleimide (Marrian, 1949). The amino groups of the carrier can be modified to an adjustable extent with an active ester (e.g., a succinimidyl ester) bearing a maleimide moiety. Subsequently the maleimido groups are allowed to react with peptides bearing a sulphydryl group (Fig. 1, route A).

Peptides devoid of sulphydryl groups (i.e., containing no cysteinyl residues) can also be thiolated in a reproducible way by functionalization of one particular amino group. Homocysteine thiolactone (Lee et al., 1980) and the succinimidyl esters of *S*-acetylthioacetic acid (SATA) (Duncan et al., 1983) and 3-(2-pyridyldithio)propionic acid (SPDP) (Carlsson et al., 1978) are examples of reagents which have been used for this purpose. Since both the peptide and the protein in the latter route are unified through acylation of amino groups it is evident but nonetheless important to note that thiolation and maleylation of carrier and peptide can be reversed. A further method of controlled conjugation involves the generation of S–S (sulphanyl or disulphide) links by the thiolysis of activated disulphides originating from the acylation of a protein or a peptide with SPDP (Fig. 1, route B).

We have investigated the effect of several coupling reagents on the antigenicity and the immunogenicity of the conjugates, taking into account the number of conjugated peptide molecules and also ensuring specific conjugation. The model peptide used was the decapeptide [Val^5]-angiotensine I and the carrier was tetanus toxoid. Three maleimide derivatives have been used to modify the carrier protein: succinimidyl *m*-maleimidobenzoate (MBS) (Kitagawa and Aikawa, 1976); succinimidyl 4-(*N*-maleimidomethyl)-*cyclo*-hexane-1-carboxylate (SMCC) (Yoshitake et al., 1979); succinimidyl 6-(*N*-maleimido)-*n*-hexanoate (MHS) (Keller and Rudinger, 1975; cf. Wünsch et al., 1985). For comparison a disulphide linked conjugate was synthesized using SPDP to modify the carrier.

Materials and methods

Angiotensin and ornithine derivatives

[Val^5]-angiotensin I (AI) was available from our organic chemistry laboratory. The decapeptide was acylated with Fmoc-Orn(Boc)-OPfp, deprotected by β-elimination of the Fmoc group using a base and further functionalized with an acetylthioacetyl group to give Ata-Orn-AI or with the maleimide derivative MHS resulting in Mh-Orn-AI. The mono-hydrochlorides of Ata-Orn-NHMe and Mh-Orn-NHMe were prepared following standard procedures.

Bifunctional reagents

Succinimidyl *m*-maleimidobenzoate (MBS) was obtained from Pierce (Rockford, U.S.A.). Succinimidyl 6-(*N*-maleimido)-*n*-hexanoate (MHS) was a gift from Boehringer (Mannheim, F.R.G.). Succinimidyl 4-(*N*-maleimidomethyl)-*cyclo*-hexane-1-carboxylate (SMCC) was synthesized with modifications according to the methods of Yoshitake et al. (1979) and Wünsch et al. (1985). Succinimidyl *S*-acetylthioacetate (SATA) was synthesized according to Duncan et al. (1983). Succinimidyl 3-(2-pyridyldithio)propionate (SPDP) was obtained from Pierce (U.S.A.).

Carriers

Tetanus toxoid (TT) and diphtheria toxoid (DT) were obtained from Dr. J. Nagel (RIVM). Tetanus toxoid contained 48 equivalent amino groups (per 150 kDa molecule) available for coupling and for diphtheria toxoid this ratio was 9 per 62 kDa. In both cases these values were determined by the 2,4-dinitrophenyl method of Sanger (reviewed by Needleman, 1970).

Conjugation method

(a) Derivatization of the carrier. The coupling method was a modification of the procedures of Lee et al. (1980) and of Green et al. (1982): 1 ml of carrier solution containing 10 mg/ml of protein was equilibrated in phosphate-buffered saline (0.1 M phosphate, pH 8.0–8.5; 0.9% NaCl) by gel filtration on PD-10 Sephadex (Pharmacia, Sweden). A bifunctional reagent (MHS, SMCC, MBS or SPDP) was added as a 1% solution in DMF at a molar ratio corresponding to the desired number of peptides to be conjugated, i.e., 1.5 equivalents of succinimidyl ester per amino group to be coupled. Following a reaction period of 5 min the reaction mixture was subjected to gel filtration using PD-10 Sephadex equilibrated in phosphate buffer (0.1 M, pH 6.66, 0.9% NaCl, 5 mM EDTA). The amount of maleimido groups coupled was determined using 5,5′-dithiobis(2-nitrobenzoic acid), DTNB, Merck (Sedlack and Lindsay, 1968). Prior to this thiol group detection the maleimido conjugates were treated with excess β-mercaptoethanol. The amount of SPDP coupled was determined by measuring the release of thiopyridone following reduction of the disulphide bond (Carlsson et al., 1978) with DTE (1,4-dithioerythritol, Fluka, Switzerland). The activated carriers were frozen and stored at −20 °C.

(b) Deprotection of the peptide. Two methods were used for deacetylation of Ata-Orn-angiotensin. The in situ method of Duncan et al. (1983) with hydroxylamine hydrochloride in a phosphate buffer (pH 6.5, 0.1 M). A faster method permitting the easy isolation of the *N*-thioacetylpeptide was performed with a hard base as used in the 'short-high' deprotection method of Msc compounds (Tesser and Balvert-Geers, 1975; Boon and Tesser, 1985). Although this procedure includes a neutralization step after about 5 s, it cannot be used in conjunction with maleyl groups and some disulphides.

(c) Coupling. Immediately after deprotection of the angiotensin derivative the peptide was added to the modified carrier in a 2 : 1 molar ratio. The reaction mixture was stirred for 1 h at room temperature. For TT modified with SPDP, the coupling was monitored by thiopyridone release with reaction times up to 24 h. The conjugated product was dialysed against PBS (0.01 M phosphate, 0.9% sodium chloride; pH 7.3).

(d) Analysis of the conjugates. All protein determinations were performed using the method of Peterson (1977). Spectrophotometric measurements were made with a Cary 118 instrument at ambient temperature. Amino acid analysis was used to determine the amount of peptide in the conjugate. Peptides were hydrolysed in sealed evacuated vials in 5.7 N hydrochloric acid (Merck, suprapur, Darmstadt, F.R.G.) at 110 °C for 24 h, carriers and conjugates under the same conditions for 48 h. Hydrolysates were analysed on a Varian LC 5000 analyser. HPLC-gel filtration on a TSK-4000 column (eluent 0.01 M phosphate; 0.1 M sodium chloride; pH 6.8; flow rate 1 ml/min) was used to analyse the peptide conjugates bearing different amounts of peptide. The antigenicity of the conjugates was tested in an ELISA. An angiotensin-TT conjugate coupled with glutardialdehyde (supplied by Vishna Kanhai, RIVM, Bilthoven) was used as a reference.

Enzyme-linked immunosorbent assay (ELISA)

ELISAs were performed in PVC microtitre plates (Flow Laboratories, Scotland). PBS with 0.01% Tween 20 (PBST) was used as the washing buffer and PBST containing 0.5% bovine serum albumin was used as the diluent for the samples and the antisera. The substrate was prepared by adding 1.67 ml of a solution of 3,3′,5,5′-tetramethylbenzidine (Pierce, U.S.A.) in dimethylsulphoxide to 100 ml citrate buffer pH 5.5. Just before use 10 μl of 30% (w/v) hydrogen peroxide was added. The antigenic activity of the conjugates was tested in an ELISA. Microtitre plates were coated with 100 μl anti-TT sheep antiserum (SATS) per well. Following overnight incubation at room temperature, the plates were washed twice. Subsequently, 100 μl of a solution containing conjugate or TT were added and the plates were incubated at 37 °C for 90 min. After washing as before rabbit anti-angiotensin antiserum (Calbiochem-Behring, La Jolla, U.S.A.) or a blank serum was added and the plates incubated for 90 min at 37 °C before a further wash stage. Finally, the plates were incubated with peroxidase-labelled sheep anti-rabbit IgG or with enzyme-conjugated sheep anti-TT serum (100 μl, 1 h, 37 °C) and washed three times. 100 μl of substrate were added and the reaction stopped by the addition of 2 M sulphuric acid (100 μl). The optical density at 450 nm was measured in a Titertek-Multiskan spectrophotometer (Flow Laboratories, Scotland).

TABLE I

ACTIVATION OF THE LYSYL GROUPS IN TETANUS TOXOID

Coupling reagent	pH	Time (min)	Ratio linker/ lysyl added	Coupling ratio	Yield [a] (%)
MHS	6.7	60	3.6	0.35	10
	6.7	60	1.8	0.22	12
	6.7	60	0.9	0.13	14
	6.7	60	0.45	0.06	14
	6.7	60	0.23	– [b]	–
	8.0	10	0.63	0.35	56
	8.5	5	0.94	0.50	53
	8.5	5	0.94	0.48	51
	8.5	5	0.52	0.38	72
	8.5	5	0.38	0.27	71
MBS	8.0	10	0.63	0.35	56
SMCC	8.0	10	0.63	0.29	46
	8.5	5	0.20	0.14	68
SPDP	8.0	10	0.63	0.31	49
	8.5	5	0.5	0.39	77
	8.5	5	0.5	0.34	69
	8.5	5	0.5	0.36	73
	8.5	5	0	0	0
	8.5	5	0.11	0.07	61
	8.5	5	0.33	0.24	71
	8.5	15	0.33	0.26	78
	8.5	5	0.90	0.62	69

[a] Yield: amount of reagent coupled compared to the amount added.

[b] Not determined, value below detection limit.

TABLE II

DETERMINATION OF THE PEPTIDE/CARRIER RATIO OF CONJUGATES

Conjugate code	SPDP added [a]	SPDP calc. [b]	SPDP determ. [c]	Amino acid	
				Mean [d]	Orn [e]
BC1	24.0	16.8	18.5	19.1	21.0
BC2	18.0	12.6	10.7	8.5	9.0
BC3	9.0	6.3	5.9	4.2	6.3
BC4	4.5	3.2	4.2	4.0	4.7
BC5	3.4	2.4	2.5	4.2	4.2

[a] Equivalents of SPDP added are corrected values, based on the succinimidyl contents.

[b] Calculated number of SPDP coupled, assuming the coupling efficiency was 70%.

[c] Number of SPDP coupled, determined by thiopyridone release following reduction with DTE.

[d] Amount of peptide coupled, determined by amino acid analysis, taking the mean of the difference between TT and conjugate (Briand et al., 1985).

[e] Amount of peptide coupled, determined by amino acid analysis, with reference to the ornithine content of the introduced peptide.

Immunization

6-week-old NIH male mice were immunized intraperitoneally with 10 μg of the conjugate, emulsified in complete Freund's adjuvant. After 6 weeks the mice were boosted with antigen in incomplete Freund's adjuvant and then bled 2 weeks later.

Immunogenicity

The antibody responses to angiotensin, spacer and carrier were determined using the ELISA. For angiotensin antibody responses, microtitre plates were precoated with 0.1% glutardialdehyde in PBS (pH 7.2, 150 μl/well) for 2 h at room temperature and washed twice. Angiotensin, its derivative, the diphtheria conjugates (see Table III) and TT were incubated overnight at room temperature (1 μg/well). The antigen solution was discarded and the plates were saturated with BSA (1% in PBST) for 1 h at room temperature. The plates were incubated with serial dilutions of antiserum. The assay was completed as described previously, using peroxidase-labelled sheep anti-mouse IgG conjugate at a 1/500 dilution.

Results

Activation of the carrier

The reaction efficiencies of the succinimidyl esters MHS, SMCC, MBS and SPDP with the available lysyl side chains in tetanus toxoid were tested by the addition of different amounts of the reagents. At pH 6.7 and using a 1 h reaction time the coupling efficiency (the fraction of the reagent molecules reacting) amounted to just 10%. Increasing the pH and lowering the reaction time permitted activation of a carrier in a predictable manner, i.e., at pH 8.5 the reactive groups were introduced with an efficiency of about 70 ± 4% within 5 min (c.f. Table I).

Loading density versus antigenicity and immunogenicity

In a preliminary experiment tetanus toxoid was thiolated with different amounts of SPDP, i.e., 0, 16, 32 and 48 mol/molTT and subsequently conjugated with Mh-Orn-angiotensin. During dialysis of the conjugates a precipitate was formed. HPLC-gel filtration of the soluble fraction confirmed that a higher peptide density leads to less soluble conjugates (Fig. 2). Conjugate I (Fig. 2*A*) modified with coupling reagent but containing no peptide showed no changes in molecular size in the main component compared to unmodified tetanus toxoid (Fig. 2*E*). From Fig. 2*B* it could be concluded that conjugate II, bearing 16 mol peptide/mol TT, contained components with molecular weights which differed from both modified (Fig. 2*A*) and unmodified (Fig. 2*E*) tetanus toxoid. The heterogeneity in the molecular weight of the tetanus toxoid, was probably related to the formaldehyde treatment used during toxoid preparation (Bizzini et al., 1970). A reference conjugate, i.e., tetanus toxoid and angiotensin coupled with glutardialdehyde, showed a similar (although less pronounced) elution profile (Fig. 2*F*). The dialysates III and IV with loading densities of 32 and 48 contained (almost) no soluble conjugate fraction. The peaks emerging with retention times of 27–28 min arose from the modification of tetanus toxoid by the coupling reagent (see Figs. 2*A* and 2*E*). The exact composition of these components was not clear although the elution profiles measured at 280 nm suggested an aromatic component (results not shown).

Testing the antigenicity of the conjugates in an ELISA using the indirect coating procedure, we found that the response diminished in spite of the increasing amount of incorporated angiotensin (Fig. 3). This phenomenon is probably an effect of the ELISA protocol: the indirect coating of the conjugate is less efficient with higher loading densities.

TABLE III

SPECIFICATION OF THE LINKER CONJUGATES USED FOR IMMUNIZATION AND DETECTION IN ELISA

Conjugate code	Hapten	Linker	Carrier	Peptide/carrier ratio		
				Reagent [a]	Orn [b]	AA [c]
C1	Ata-Orn-NHMe	MHS	TT	17	17	
C2	Ata-Orn-NHMe	SMCC	TT	14	20	
C3	Ata-Orn-NHMe	MBS	TT	17	12	
C4	Ata-Orn-NHMe	SPDP	TT	15	19	
C5	Ata-Orn-AI	MHS	TT	16	19	11
C6	Ata-Orn-AI	SMCC	TT	13	16	12
C7	Ata-Orn-AI	MBS	TT	17	16	15
C8	Ata-Orn-AI	SPDP	TT	13	13	10
C9	Ata-Orn-NHMe	MHS	DT	7	6	
C10	Ata-Orn-NHMe	SMCC	DT	3	5	
C11	Ata-Orn-NHMe	MBS	DT	7	4	
C12	Ata-Orn-NHMe	SPDP	DT	5	8	

[a] Amount of peptide coupled determined by measurement of the incorporation of the functional group.
[b] Amount of peptide coupled determined by amino acid analysis, based on the number of ornithyl residues.
[c] Amount of peptide coupled determined by amino acid analysis according to the method of Briand et al. (1985).

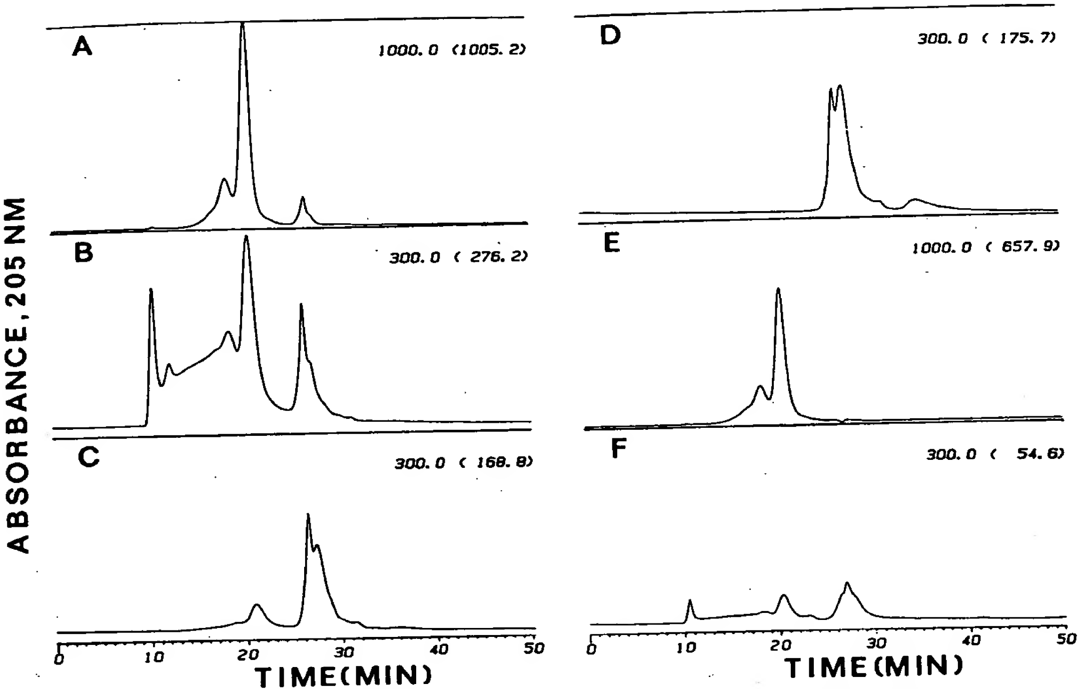


Fig. 2. HPLC gel filtration-elution profiles for angiotensin-TT conjugates with loading densities of 0, 16, 32 and 48 mol peptide/mol TT (Figs. 2*A*–2*D* resp.) compared to TT (Fig. 2*E*) and an AI-TT conjugate coupled with glutardialdehyde (Fig. 2*F*). Figs. 2*A* and 2*E* were recorded at an attenuation of 1000 mAU; Figs. 2*B*, 2*C*, 2*D* and 2*F* at an attenuation of 300 mAU. The absorbances of the peak maxima are indicated in brackets.

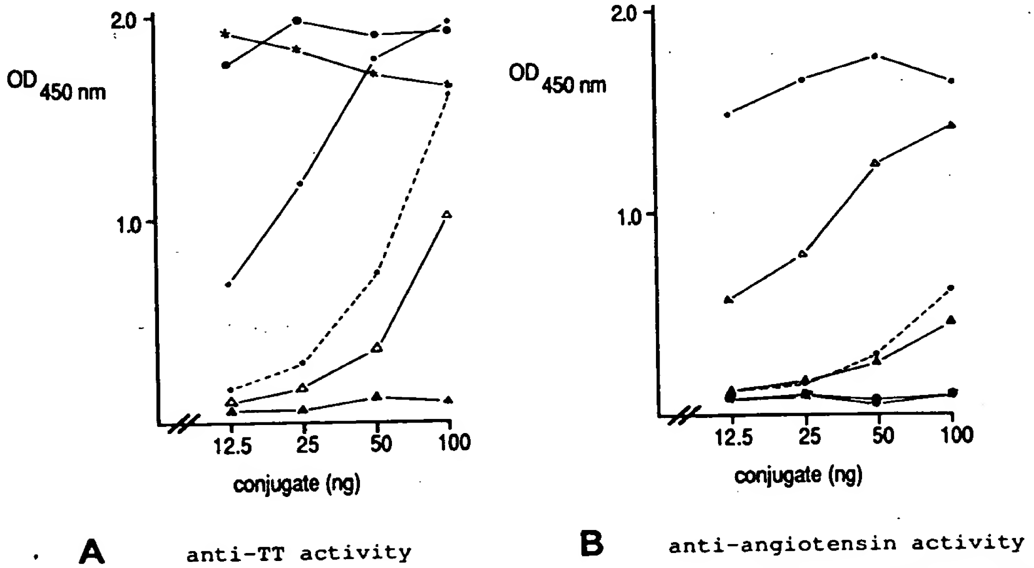


Fig. 3. Antigenicity of AI-TT conjugates with various loading densities: 0 (•—•—•); 16 (.–.–.); 32 (△–△–△); 48 (▲–▲–▲) mol AI/mol TT, compared to TT (∗–∗–∗) and an AI-TT conjugate coupled with glutardialdehyde (.—.—.), tested in an ELISA with anti-TT (*A*) and anti-angiotensin (*B*) antisera. The amount of conjugate given is the amount added per well. Preparation of the antigen: 10 μg conjugate or TT was dissolved in 8 M urea. After 1 h the solution was diluted to 10 ml with water. A serial dilution of antigen (100 μl) in buffer was added to the wells and the ELISA was performed as described in the materials and methods section.

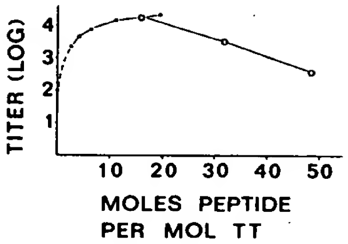


Fig. 4. The immunogenicity of angiotensin-TT conjugates with various loading densities. ELISA titres from two experiments are shown, one in the 'non-soluble' conjugate range (○——○), the other in the 'soluble' conjugate range (■——■) (see text). Titre is expressed as the reciprocal of the antiserum dilution which resulted in 50% of maximal absorption. Blood samples were taken 56 days after the first immunization. A pool of sera from eight mice were examined for each conjugate. Titres were determined in duplicate.

Taking the non-loaded conjugate as a reference, the immunogenicity of the conjugates, as measured by antipeptide antibody production, decreased with *in*creasing antigen density (Fig. 4). This was shown to be due to decreasing solubility in an experiment in which loading ratios in the 'soluble conjugate' range (comprising 2.5, 4, 6, 11 and 19 peptide units per 150 kDa of carrier (Table II)) gave rise to high antibody titres (Fig. 4). The titre of the glutardialdehyde conjugate (see materials and methods section) was comparable with the titre of the conjugate with the highest density (48 mol peptide/mol TT): 2.4 in log units.

Specificity and cross-reactivity

Four linkers were compared for their effect on the immunogenicity of the conjugate, namely three maleimide derivatives and one activated disulphide. The reagents differ through the nature of the links exerted (Fig. 1). It is supposed that there would be a negligible contribution to the diversity of the antibodies from the small structural difference in the link between peptide and carrier arising from the reversal of maleylation and thiolation of peptide and carrier.

The effect of the spacer on the immunogenicity of the resulting conjugate was investigated in an ELISA by coating the microtitre plates with conjugates consisting of an unrelated carrier protein (i.e., diphtheria toxoid), and a 'false' peptide (ornithine methylamide, Orn-NHMe). Ornithine methylamide was functionalized with SATA to give Ata-Orn-NHMe. Immunization was performed with Ata-Orn-angiotensin coupled to tetanus toxoid, and with the corresponding conjugate of Ata-Orn-NHMe (Table III). In Table IV the immunogenicity of the conjugates is illustrated. All four angiotensin conjugates elicited antibodies against the peptide and the peptide derivative to a large extent. Anti-tetanus toxoid

TABLE IV

TITRES REPRESENTING IMMUNOGENICITY OF ANGIOTENSIN-TETANUS TOXOID CONJUGATES COMPARED TO METHYLAMIDE-TETANUS TOXOID CONJUGATES

Titre was defined as the reciprocal of the dilution of antiserum which resulted in 50% of maximal absorption. The composition of the conjugates is specified in Table III.

Immunogen	Anti-X, X =						
Tetanus Toxoid conjugates (linker)	Peptide derivatives		Diphtheria conjugates (linker)				Carrier
	AI	AtaOrnAI	C9 (MHS)	C10 (SMCC)	C11 (MBS)	C12 (SPDP)	TT
C1 (MHS)	550	1000	$>10^5$	$>10^5$	$>10^5$	$>10^5$	$>10^6$
C2 (SMCC)	850	1000	$>10^5$	$>10^5$	$>10^5$	$>10^5$	$>10^6$
C3 (MBS)	750	900	40000	36000	$>10^5$	55000	$>10^6$
C4 (SPDP)	630	670	270	320	320	700	$>10^6$
C5 (MHS)	140000	160000	3400	280	210	225	$>10^6$
C6 (SMCC)	96000	100000	9700	54000	1800	245	$>10^6$
C7 (MBS)	155000	200000	550	660	$>10^5$	260	$>10^6$
C8 (SPDP)	16500	40000	150	180	320	370	$>10^6$

titres were high (> 10^6) despite modification of the tetanus toxoid carrier. In the case of all conjugates except the SPDP-derived C4 and C8 conjugates there was a high antibody response to the homologous linker conjugate. However, cross-reactions occurred, especially with the anti-blank conjugate antibodies elicited by C1 (MHS), C2 (SMCC) and C3 (MBS). The anti-angiotensin conjugate antibodies showed cross-reactions to a much lesser extent and there were few cross-reacting antibodies elicited by C5 (MHS) and C8 (SPDP).

Discussion

Reaction conditions

Lee et al. (1980) reported that MHS reacted with TT with a high efficiency until 15 maleimido groups per 10^5 Da had been introduced. The reaction ran at pH 6.7 and required 60 min but for higher densities an excess of MHS had to be added. In order to increase the reaction rate, the pH of the reaction mixture should be increased: Boon (1985) found that the reaction of 2-(methylsulphonyl)ethyl succinimidyl carbonate (Msc-ONSu) with the ϵ-amino groups of the (19) lysyl residues in cytochrome *c* was completed within 5 min at pH 8.5 and showed that the reaction proceeded stoichiometrically. Hydrolysis of the active ester was relatively slow (see also Aldwin and Nitecki (1987) and Anjaneyulu and Staros (1987)). In the present work it has been demonstrated that if the reaction is performed at a higher pH, it is possible to obtain a high density of maleimido groups without the requirement for a large excess of MHS. However, the ‘spontaneous deterioration’ of the succinimidyl ester moieties in the reagents of Fig. 1 should be taken into account and their actual activity should be determined beforehand.

The maleimido group of MHS proved to be 99% stable for at least 15 min at pH 8.5, confirming the observation of Wünsch et al. (1985). The maleimido group in MBS is less stable and therefore the activation was performed at pH 8.0 with a molar ratio of MBS/amino group of 2. Subsequent gel filtration of the reaction mixture should be carried out at pH 6.7. Lee et al. (1980) have reported that the reaction proceeds in the absence of thiol groups with an efficiency of only 10% over 30 min and therefore the possibility of nucleophilic addition of amino groups can be discounted. Storage of the modified carrier at −20 °C is recommended to slow down any functional deterioration catalyzed by imidazolyl functions occurring in the protein (His). At −20 °C the activated carrier is stable for at least a few months. At 4 °C the thiol binding activity decreases with a halftime of about 1 month.

Loading density

The concentration of cross-linking reagent during activation of the carrier determines the loading of the carrier only to a minor degree. Thus at a given pH the loading density appears only to depend on time. This effect is to be expected since the pH determines the number of reactive amino groups (p*Ka* ϵ-amino group = 10.5); this quantity is far outnumbered by the amount of succinimidyl ester molecules so the reaction appears to run at a given pH with near zero-order kinetics (Table I).

Determination of carrier load from amino acid analysis (cf. Briand et al., 1985) was found to be less accurate than from the number of maleimido groups introduced per molecule of carrier (cf. Lee et al., 1980). The latter method was particularly suitable when large peptides were to be coupled to a protein-carrier and the amino acid composition resembled that of the carrier. The intentional introduction of a diagnostic amino acyl group (Orn and Nle) was also another efficient method. Carrier modification is a stoichiometric reaction: reduction of the reaction time to 5 min at pH 8.5 diminishes the efficiency to about 70%.

Experiments with angiotensin I as a model peptide and tetanus toxoid as the carrier protein showed that an optimum loading density exists which amounts to 10–20 peptides/mol tetanus toxoid. This number is somewhat lower than that reported by Stevens et al. (1981) (25/10^5 kDa tetanus toxoid) but the curve is truncated by the insolubility of the higher substituted carrier molecules (in our case at a molar ratio of 32 and higher). Stevens et al. apparently had no solubility problems. Masking of determinants could be an explanation for decreasing immunogenicity at increasing densities. If the anti-TT response is measured following urea denaturation (to effect solubility), the TT-response decreases with increasing density, whereas the angiotensin response decreases only slightly. Masking of carrier determinants could be the explanation of this phenomenon but masking of hapten determinants is also possible since closely packed angiotensin residues would impair recognition by the immune system.

Specificity and cross-reactivity

The effect of several bifunctional reagents on the immunogenicity of the conjugate was investigated. MHS, MBS, SMCC and SPDP were used as cross-linkers in peptide-protein conjugation with angiotensin I as the peptide and tetanus toxoid as the carrier protein. The results showed that the immunogenicity of a glutardialdehyde conjugate was comparable to that of a highly substituted sulphur-linked conjugate. Since an excess of glutardialdehyde was routinely used, a reference conjugate was not included when the low density sulphur-linked conjugates were investigated.

The polyclonal antisera raised against the angiotensin conjugates included antibodies which reacted specifically with the unmodified decapeptide angiotensin I in an ELISA. There was no significant difference in reactivity with an angiotensin coat or with derivatized angiotensin I and titres ranged from 2×10^4 to 2×10^5.

The reactivity of the antisera towards tetanus toxoid was also very high, of the order of 10^6, irrespective of the coupling of the peptide. This suggests that the character of this carrier, chosen since it can be used for human immunization, was not changed profoundly.

Four [angiotensin]-linker-[tetanus toxoid] conjugates were used to raise antibodies. The antisera also contained antibodies directed against the linkers, since they cross-reacted with their homologous [ornithine methylamide]-linker-[diphtheria toxoid] conjugates. There were however, qualitative differences: (1) the SPDP spacer containing an aliphatic disulphide, showed almost no reactivity; (2) for the MHS linker, which results in a flexible aliphatic chain connected through a disulphide bridge to a succinimide ring, the antibody level was somewhat higher; (3) the rigid linkers produced by SMCC and MBS, containing an additional cycloaliphatic or aromatic ring, induced very high antibody titres, within the range 10^4–10^5.

It has previously been shown that cross-linkers such as glutardialdehyde, carbodiimide or reagents containing an aromatic moiety elicit antibodies directed against the spacer (Palfreyman et al., 1984; Briand et al., 1985; Bernatowicz and Matsueda, 1985). Aldwin and Nitecki (1987) demonstrated by Western blot analysis that peptide-protein conjugates cross-linked with a water-soluble active ester of 6-(*N*-maleimido) *n*-hexanoic acid did not induce detectable antibody specific for the spacer. The spacer originating from MHS used in the present study was the same as that investigated by Aldwin and Nitecki and our results confirm their findings quantitatively. The cross-linking agents MHS and SPDP are preferable to SMCC and MBS in terms of their lower potential for immunogenicity, greater flexibility and greater stability in aqueous solutions. A drawback of SPDP coupling is the resulting disulphide linkage, which confers a susceptibility to reductive cleavage by ubiquitously occurring thiol compounds. The thio-ether linkage resulting from the application of MHS is very stable (at least for 6 months at pH 6 at 4°C) (Yoshitake et al., 1982) and we conclude that MHS is the bifunctional reagent of choice for coupling peptides to proteins.

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A 50-kDa fragment from the NH_2-terminus of the heavy subunit of *Clostridium botulinum* type A neurotoxin forms channels in lipid vesicles

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1. A 50-kDa fragment representing the NH_2-terminus of the heavy subunit of botulinum type A neurotoxin was found, at low pH, to evoke the release of K^+ from lipid vesicles loaded with potassium phosphate. Similar K^+ release was also observed with the intact neurotoxin, its heavy chain and a fragment consisting of the light subunit linked the 50-kDa NH_2-terminal heavy chain fragment. The light subunit alone, however, was inactive.

2. In addition to K^+, the channels formed in lipid bilayers by botulinum neurotoxin type A or the NH_2-terminal heavy chain fragment were found to be large enough to permit the release of NAD (M_r 665).

3. The optimum pH for the release of K^+ was found to be 4.5. Above this value K^+ release rapidly decreased and was undetectable above pH 6.0.

4. The binding of radiolabelled botulinum toxin to a variety of phospholipids was assessed. High levels of toxin binding were only observed to lipid vesicles with an overall negative charge; much weaker binding occurred to lipid vesicles composed of electrically neutral phospholipids.

5. A positive correlation between the efficiency of toxin-binding and the efficiency of K^+ release from lipid vesicles was not observed. Whereas lipid vesicles containing the lipids cardiolipin or dicetyl phosphate bound the highest levels of neurotoxin, the toxin-evoked release of K^+ was low compared to vesicles containing either phosphatidyl glycerol, phosphatidyl serine or phosphatidyl inositol.

6. The implications of these observations to the mechanism by which the toxin molecule is translocated into the nerve ending are discussed.

The neurotoxin of *Clostridium botulinum* type A (BoNT/A) is a 145-kDa protein which acts primarily at the neuromuscular junction causing muscular paralysis by inhibiting the release of the neurotransmitter acetylcholine [1, 2]. The toxin is a two-chain molecule comprising a light subunit (55 kDa) linked by a disulphide bridge to a heavy subunit (95 kDa) [3]. The neuroparalytic activity of BoNT/A is thought to be accomplished in at least three stages: an initial binding stage, an internalisation stage and then one or more steps which disable the acetylcholine release mechanism. BoNT/A has been shown to bind specifically to acceptors on rat brain synaptosomes with high affinity (K_d = 0.6 nM) by an active-site region located on the heavy subunit which is believed to bind the toxin to the presynaptic surface of cholinergic nerve endings prior to internalisation [4, 5]. Little is presently known about the mechanism by which the toxin is internalised. The process is energy-requiring and may resemble the process of receptor-mediated endocytosis [5]. The molecular mechanism by which the toxin inhibits transmitter release is still completely unknown.

Botulinum toxin is structurally similar to both tetanus and diphtheria toxins and for both the latter toxins it has been demonstrated that an NH_2-terminal portion of the larger subunit induces pore formation in lipid bilayers, a property which may be relevant to the transport of an active toxin fragment into the cytoplasm [6–8]. Using planar lipid bilayers, similar pore formation by both botulinum type C_1 neurotoxin [9] and the heavy subunit of botulinum type B neurotoxin has also been demonstrated [10].

In this study, using liposomes of defined phospholipid composition, it is shown that BoNT/A is capable of forming channels in lipid bilayers at low pH. It is further demonstrated that this channel-forming activity is retained by a 50-kDa fragment representing the NH_2-terminal portion of the heavy subunit. The properties of the lipid-toxin interaction are examined and discussed in relation to a possible mechanism for toxin internalisation.

MATERIALS AND METHODS

Materials

Phosphatidyl glycerol, phosphatidyl ethanolamine, phosphatidyl serine, phosphatidyl inositol, soybean phosphatidyl choline (type IIS), cardiolipin, dicetyl phosphate and gramicidin were obtained from the Sigma Chemical Company. Phosphatidyl choline (lecithin grade I) and urea (Aristar

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Abbreviations. BoNT/A, *Clostridium botulinum* type A neurotoxin; mouse LD_{50}, amount of toxin that killed 50% of mice injected (i.p.) with toxin; PtdCho, phosphatidyl choline; PtdGro, phosphatidyl glycerol; PtdEtn, phosphatidyl ethanolamine; PtdIns, phosphatidyl inositol; PtdCho/PtdGro vesicles, liposomes consisting of equimolar proportions of PtdCho and PtdGro.

Enzyme. Trypsin (EC 3.4.21.4).

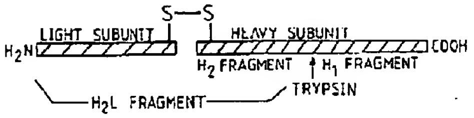

Fig. 1. Clostridium botulinum *type A neurotoxin*

grade) were supplied by BDH Chemicals Ltd. [*carbonyl*-^{14}C]NAD and [^{125}I]iodine were obtained from Amersham International.

Purification of Clostridium botulinum *type A neurotoxin and its tryptic fragments*

C. botulinum type A neurotoxin was purified to a specific toxicity of $1-2 \times 10^8$ mouse LD_{50} mg^{-1} by affinity chromatography on *p*-aminophenyl-*β*-D-thiogalactopyranoside as described previously [11]. The H_2L fragment (see Fig. 1) (specific toxicity $6-12 \times 10^3$ mouse LD_{50} mg^{-1}) was purified by a modification of a previously described procedure [11]. Botulinum neurotoxin (40 mg in 15 ml) in 0.15 M Tris/HCl buffer, pH 8.0 containing 100 mM NaCl was treated with trypsin (50 μg ml^{-1}) for 72–96 h at 20°C, dialysed against 20 mM triethanolamine buffer, pH 7.8 containing 100 mM NaCl, filtered (0.22 μm pore size) and then chromatographed (fast protein liquid chromatography system, Pharmacia) on a Mono Q column (HR 10/10, Pharmacia) equilibrated in the latter buffer. The column was washed with a further 100 ml of the triethanolamine buffer and then the H_2L fragment eluted with triethanolamine buffer (20 mM, pH 7.8) containing 200 mM NaCl. The purified fragment (≈ 1.5 mg ml^{-1}) was made 5 μg ml^{-1} with trypsin inhibitor, dialysed against 0.15 M Tris/HCl buffer, pH 8.0 containing 50 mM NaCl and stored at -20°C. The heavy chain and H_2 fragment of botulinum type A neurotoxin were purified from the neurotoxin and H_2L fragment respectively by precipitating the light subunit in the presence of 2.5 M urea, 1 M NaCl and 100 mM dithiothreitol as described previously [11]. The light subunit of botulinum type A neurotoxin (< 100 mouse LD_{50} mg^{-1}) was purified from the H_2L fragment by the method of Kozaki et al. [12].

Preparation of liposomes and K^+ release studies

Lipid vesicles were prepared essentially by the method of Enoch and Strittmatter [13]. Phospholipid mixtures (60 μmol total lipid) were suspended in 3 ml 0.1 M potassium phosphate buffer, pH 7.2 containing 1 mM EDTA and 10 mM sodium deoxycholate and then briefly sonicated (three 5-s periods, Dawe Instrument type 7530 A, setting 3) in ice under nitrogen gas. Sodium deoxycholate was removed from the liposomes by dialysis three times against 500 ml 0.1 M potassium phosphate buffer, pH 7.2 containing 1 mM EDTA at 4°C. Soybean phospholipids were first washed with acetone prior to liposome preparation [14]. Liposomes made with 0.1 M potassium acetate buffer pH 4.5 were prepared by the freeze-thaw technique of Kasahara and Hinkle [14].

Detection of K^+ release from lipid vesicles was carried out essentially as described by Boquet and Duflot [8]. Lipid vesicles (50 μl) were added to 15 ml of the appropriate buffer and slowly stirred. Potassium release was monitored using a Philips K^+ electrode connected via Philips model 9421 pH meter to an Oxford Instruments 3000 chart recorder.

Release of [^{14}C]NAD from liposomes

For studying the release of [^{14}C]NAD, liposomes were prepared as above with 3 ml 0.1 M sodium phosphate buffer, pH 7.2 containing 1 mM EDTA and [^{14}C]NAD (37 kBq ml^{-1}) using half the concentration (5 mM) of sodium deoxycholate. The detergent was then removed by gel filtration on a column of Sephadex G-50 (25 × 3 cm) equilibrated in potassium phosphate buffer pH 7.2 containing 1 mM EDTA. Liposomes, eluted in the void volume, were then dialysed against 1 l of the latter phosphate buffer. Release of NAD was assessed with 1.5-ml portions of liposome preparations. After diluting to 11.5 ml with 0.1 M sodium acetate buffer, pH 4.3 (to give a final pH of 4.5) either buffer, toxin of Triton X-100 was added and the mixture incubated for 30 min at 20°C before being centrifuged at 200000 × *g* for 1 h. Portions (1 ml) of the supernatant fluids were removed and their radioactivity assessed by scintillation counting.

Binding of radioiodinated botulinum neurotoxin to liposomes

Botulinum neurotoxin, radioiodinated using chloramine-T [4], was mixed with unlabelled toxin to give a specific activity of 1.7 MBq mg^{-1} and further diluted with 0.1 M sodium phosphate buffer, pH 7.2 containing 1 mg ml^{-1} ovalbumin to a neurotoxin concentration of 100 μg (0.17 MBq) ml^{-1}. Liposomes (0.5 ml) prepared as described above were mixed with 11 ml 0.1 M sodium acetate buffer, pH 4.4 and 100 μl of the radiolabelled toxin solution, incubated for 30 min at 20°C and then centrifuged at 200000 × *g* for 1 h. The supernatant fluid was carefully removed and the excess fluid allowed to drain from the liposome pellet by inverting the tubes for 5 min. The liposomes were resuspended in 1 ml 0.1 M sodium phosphate buffer, pH 7.2 and the radioactivity in 100-μl portions measured.

RESULTS

Release of K^+ from phosphatidyl choline/phosphatidyl glycerol vesicles

Addition of 10 μg of the channel-forming polypeptide gramicidin to phosphatidyl choline/phosphatidyl glycerol (molar ratio, 1:1) liposomes (PtdCho/PtdGro vesicles) loaded with K^+ caused a rapid (< 30 s) releases of K^+ at pH 4.0 (Fig. 2a). No further release of K^+ was detected when an additional 10 μg gramicidin was added, indicating that all the available K^+ had been released. An equivalent quantity of K^+ was liberated at pH 7.2 showing that the low pH had not affected the integrity of the vesicles.

Addition of increasing amounts of the H_2 fragment of botulinum type A neurotoxin to PtdCho/PtdGro vesicles suspended in buffer at pH 4.0 evoked the release of K^+ ions (Fig. 2b and c). Under the experimental conditions, the addition of 1 nmol of the H_2 fragment released all of the available K^+ in less than 1 min; addition of gramicidin at this point caused no further release of K^+. Treatment of the vesicles with a further portion of the H_2 fragment after gramicidin treatment had no effect of the baseline value. At pH 7.2 the H_2 fragment was completely ineffective at releasing K^+ from vesicles (Fig. 2d).

Analogous experiments conducted with the intact neurotoxin, heavy chain and H_2L fragment of BoNT/A yielded qualitatively similar results. The results for the intact toxin are shown in Fig. 3a–c. At pH 4.0, BoNT/A, the H_2L

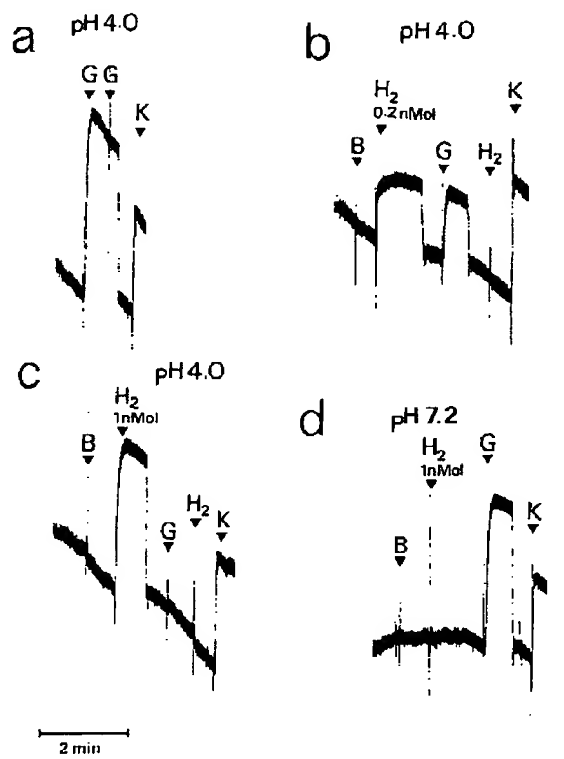

Fig. 2. *Release of K^+ from PtdCho/PtdGro vesicles by the H_2 fragment of BoNT/A.* (a) After addition of vesicles to 0.1 M sodium acetate buffer, pH 4.0, gramicidin (G, 10 µl of 1 mg ml^{-1} solution in ethanol) was added. When maximal response had been obtained, the baseline was adjusted to zero and KCl (K, 10 µl of a 20 mM KCl solution) was added as a standard. (b, c) After the addition of the fragment buffer as a control (B), the H_2 fragment was added to vesicles in 0.1 M sodium acetate buffer, pH 4.0, at the indicated amounts. At maximal response, the baseline was returned to zero and gramicidin added as above (G) followed by baseline adjustment and further addition of the same amount of the H_2 fragment. Finally KCl standard was added as in (a). (d) The H_2 fragment was added to vesicles in 0.1 M sodium phosphate buffer, pH 7.2; additions of buffer (B), gramicidin (G) and KCl (K) were as described in (b). The volume of the reaction mixture in each case was 15 ml

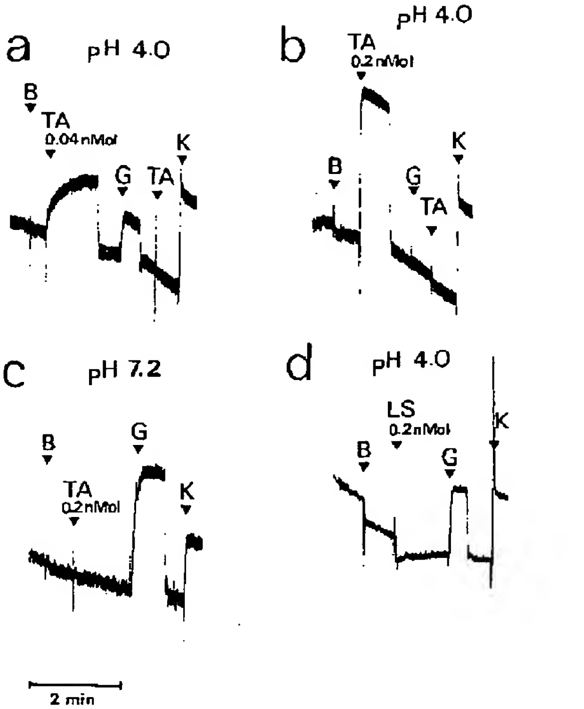

Fig. 3. *Release of K^+ from PtdCho/PtdGro vesicles by BoNT/A and its light subunit.* (a, b) BoNT/A (TA) was added to vesicles in 0.1 M acetate buffer, pH 4.0, at the indicated amounts. (c) BoNT/A was added to vesicles in 0.1 M sodium phosphate buffer, pH 7.2. (d) Light subunit (LS) was added to vesicles in acetate buffer pH 4 at the indicated amount. Baseline adjustments and additions of the fragment buffer control (B), gramicidin (G) and KCl (K) were as described in Fig. 2

fragment and the heavy chain were found to be more efficient at releasing K^+ from PtdCho/PtdGro vesicles than the H_2 fragment; on a molar basis 4–5-fold less of the heavy chain, H_2L fragment and intact toxin consistently gave the same release of K^+ as the H_2 fragment. At pH 4.5, however, H_2 fragment was equally as effective at releasing K^+ from lipid vesicles as the intact neurotoxin (see below). As found for the H_2 fragment, at pH 7.2 intact toxin (Fig. 3c) heavy subunit and H_2L fragment did not release K^+ from lipid vesicles.

The light subunit of BoNT/A was ineffective at releasing K^+ from lipid vesicles at both pH 4.0 (Fig. 3d) and pH 4.5. BoNT/A which had been inactivated with formaldehyde retained less than 3% of the K^+-releasing activity of the native neurotoxin.

Similar results were obtained when crude soybean phospholipid vesicles were substituted for the PtdCho/PtdGro vesicles. The amount of the neurotoxin and its fragments required to release all the K^+ from soybean lipid vesicles, however, had to be increased by 3–5-fold over that required for PtdCho/PtdGro vesicles.

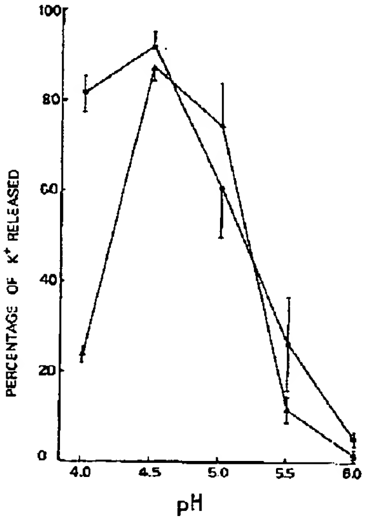

Fig. 4. *Effect of pH on the release of K^+ from PtdCho/PtdGro vesicles by BoNT/A and its H_2 fragment.* Liposomes (50 µl) were suspended in 15 ml 0.1 M sodium citrate/sodium phosphate buffer at each pH and 0.2 nmol of either BoNT/A (●) or the H_2 fragment (▲) added. The amount of K^+ released was assessed by measuring the response 80 s after addition of the toxin and expressing this value as a percentage of the response obtained with the gramicidin control at the same pH value. Values represent the mean of at least five measurements

Table 1. *Release of [^{14}C]NAD from PtdCho/PtdGro vesicles by BoNT/A or its H_2 fragment at pH 4.5*
The amount of NAD released after disruption of the liposomes with Triton X-100 was taken as 100% release

Treatment of liposomes	Total ^{14}C in the supernatant fluid		Average NAD released
	1	2	
	cpm		%
Control (no addition)	6520	7394	49.6
Intact BoNT/A (2 nmol total)	11994	12914	88.7
H_2fragment (2 nmol total)	10764	11741	80.2
Triton X-100 (1% final concn)	13328	14731	100

Table 2. *Binding of ^{125}I-BoNT/A to, and release of K^+ from liposomes of different phospholipid composition*
For the assessment of BoNT/A binding each incubation mixture (11.5 ml) contained 10 µg neurotoxin (17 kBq) and 10 µmol lipid. The percentage of K^+ released by 0.2 nmol BoNT/A from liposomes in 0.1 M sodium acetate buffer, pH 4.5 was assessed as described in Fig. 4

Phospholipid composition of liposomes (molar ratio)	^{125}I-BoNT/A bound	K^+ released
	% total	%
PtdCho/PtdGro (1:1)	51 ± 5.0	> 95
Soybean	71 ± 4.5	63 ± 16
PtdCho	4.0 ± 0.6	< 5
PtdCho/PtdEtn (1:1)	10.5 ± 4.0	< 5
PtdCho/PtdIns (1:1)	65 ± 2.4	> 95
PtdCho/PtdSer (1:1)	46 ± 7.0	> 95
PtdCho/cardiolipin (2:1)	80.5 ± 9.7	31 ± 6.6
PtdCho/dicetylphosphate (2:1)	90 ± 4.3	< 5
Negative control	3.3 ± 1.8	–

Effect of pH on the release of K^+ from lipid vesicles

At extra-vesicular pH values between 4.5 and 6.0, the extent to which K^+ was released from PtdCho/PtdGro vesicles was similar for both the intact botulinum neurotoxin and its H_2 fragment. Maximal release occurred at pH 4.5 (Fig. 4): above pH 4.5 the K^+ release rapidly decreased and was undetectable above pH 6.0. At pH 4.0, however, while the K^+ release by the intact neurotoxin was only slightly lower than that observed at pH 4.5, an almost fourfold reduction in K^+ release by the H_2 fragment was observed.

Changes in the pH value of the intra-vesicular environment did not appear to affect significantly the release of K^+ from lipid vesicles. Liposomes containing 0.1 M potassium acetate buffer at pH 4.5 instead of potassium phosphate buffer at pH 7.2 were found to release similar amounts of K^+ when the extravesicular environment was reduced to pH 4.5 in the presence of botulinum toxin.

Release of [^{14}C]NAD from PtdCho/PtdGro vesicles

At pH 4.5, addition of either BoNT/A or the H_2 fragment to PtdCho/PtdGro vesicles loaded with [^{14}C]NAD released over 80% of the total radioactivity (Table 1). Values obtained for the release of NAD in the presence of the neurotoxin consistently were close to double those of control values obtained in the absence of toxin, indicating that the membrane channels formed by BoNT/A are large enough to permit the release of NAD molecules (M_r 665).

Binding of ^{125}I-BoNT/A to and release of K^+ from liposomes of different phospholipid composition

At pH 4.5, the highest levels of ^{125}I-labelled BoNT/A binding were observed to liposomes comprising phospholipids with a net negative charge; liposomes incorporating either PtdGro, PtdIns or PtdSer bound approximately 50% of the total toxin and those including either dicetyl phosphate or cardiolipin, over 80% of the total toxin (Table 2). In contrast, much lower toxin binding was observed to liposomes made with the neutral phospholipids PtdCho and PtdEtn. At pH 7.2, however, less than 7% of the radiolabelled toxin was recovered in the liposome pellet regardless of the phospholipid composition. The binding of botulinum toxin to PtdCho/PtdGro vesicles was found to be partially reversible in that 45% of the toxin bound to the liposomes at pH 4.5 could be released when the pH was raised to 7.2. Iodination of BoNT/A did not affect the pore-forming properties of the toxin. Neurotoxin iodinated with increased concentrations of iodine, resulting in approximately 4 mol iodine/mol toxin, still retained more than 90% of the channel-forming ability of the untreated controls.

Whereas low levels of toxin binding to liposomes were associated with poor release of K^+ from the lipid vesicles, high levels of binding were not always accompanied by efficient K^+ release. Thus, although lipid vesicles prepared from either dicetyl phosphate or cardiolipin appeared to display the highest affinity for the neurotoxin, toxin-associated release of K^+ from these vesicles was low compared to that from vesicles comprising either PtdGro, PtdSer or PtdIns. Similarly, compared to lipid vesicles containing the latter three phospholipids, the toxin-induced release of K^+ from soybean phospholipid vesicles was significantly lower even though all these liposome preparations bound comparable amounts of toxin (Table 2).

DISCUSSION

The molecular details of the events leading to internalisation of botulinum toxin (or an active toxin fragment) into the cytoplasm of presynaptic nerve cells are only vaguely understood. Acceptor molecules present on the presynaptic nerve surface bind tightly to the molecules of neurotoxin which then cross the outer plasmalemma by an energy-dependent process resembling receptor-mediated endocytosis [5]. The mechanism by which the toxin penetrates the lipid bilayer, whether it be the membrane of the plasmalemma or an endosomal vesicle, however, is still unresolved. Lipid bilayers in the form of liposomes provide a convenient model for studying these membrane-toxin interactions. In the present study we show that a 50-kDa NH_2-terminal fragment (H_2) of BoNT/A is able to form channels in unilamellar vesicles at low pH which are large enough to allow the release of K^+ and NAD^+. Similar channel-forming activity was also observed with the intact neurotoxin, its heavy subunit and H_2L fragment. This property of the neurotoxin appears to be confined to the heavy subunit since no channel-forming activity was evident in purified samples of the neurotoxin light subunit. In addition, the similarity observed between the pore-forming activities of the heavy subunit and the H_2L fragment suggests that H_1 fragment of the neurotoxin has little or no channel-forming activity compared to the H_2 fragment.

The pH optimum of 4.5 for membrane channel formation observed for type A neurotoxin is close to values obtained in similar studies with diphtheria toxin [7] and tetanus [8] and botulinum type B neurotoxins [10]. Botulinum type C_1 neurotoxin, however, has recently been reported to form channels in planar lipid bilayers at a higher pH optimum of 6.1 [9]. One explanation for the low pH requirement of type A neurotoxin for membrane channel formation is that as the pH is lowered a hydrophobic site is exposed on the H_2 component which facilitates membrane binding. Supportive of this proposal is the observation that radiolabelled botulinum neurotoxin binds strongly to PtdCho/PtdGro vesicles only at low pH. The exposure of a hydrophobic region, induced by protein conformation changes at low pH, has also been proposed to explain the pH-dependent insertion of diphtheria [15, 16] and tetanus toxin [8] into lipid bilayers. Whatever the mechanism of pore formation for BoNT/A, a pH gradient across the membrane does not appear to be required since reducing the intra-liposomal pH from 7.2 to 4.5 did not affect the toxin-induced release of K^+.

Radiolabelled botulinum type A neurotoxin, at low pH, bound to a variety of liposomal phospholipids. High levels of toxin binding, however, were observed only to liposomes comprising phospholipids with an overall negative charge; liposomes consisting of electrically neutral lipids displayed a much weaker interaction with the toxin. The binding of neurotoxin to lipid vesicle membranes was not always accompanied by the formation of membrane channels. While the toxin bound strongly to lipid vesicles consisting of either soybean phospholipids, cardiolipin or dicetyl phosphate, the release of K^+ from these vesicles was significantly less than that observed from liposomes consisting of either PtdGro, PtdIns or PtdSer which appeared to bind the toxin less strongly. These observations may indicate that toxin binding and channel formation are two distinct events in which case some phospholipids, while allowing the toxin molecule to bind, may inhibit the subsequent channel-forming stage. A similar mechanism has been suggested for botulinum type C_1 neurotoxin for which it has been suggested that aggregates of the toxin may be involved in channel formation [9]. Alternatively, it could be argued that the neurotoxin is able to bind the lipid bilayer in several configurations, not all of which are conducive to the formation of a membrane channel. Whether the toxin binds in a channel-forming configuration or not could, then, be influenced by the nature of the membrane phospholipids. The lack of a positive correlation between toxin binding and pore formation suggests that a specific toxin conformation is required before membrane channels can form and argues strongly against the possibility that the release of K^+ from liposomes is simply due to a non-specific disruption of the integrity of the membrane surface as the toxin binds. The inability of formalin-inactivated toxin to release K^+ from liposomes and the reduced channel-forming activity of the H_2 fragment at pH 4.0 also support the view that there is a requirement for a specific toxin conformation to enable the formation of membrane pores.

Whether or not the channel-forming activity observed for the H_2 fragment of BoNT/A plays a significant role in the neuroparalytic activity of the neurotoxin is uncertain. The ability to form membrane pores at low pH has been found to be a common characteristic of a number of bacterial toxins; in addition to the other botulinum neurotoxins so far studied (types B [10] and C_1 [9]), similar channel-forming activities have been demonstrated in fragments analogous to the H_2 fragment of BoNT/A in both tetanus [8] and diphtheria [7] toxins. In the case of diphtheria toxin direct evidence has recently been obtained that illustrates the importance of a low extravesicular pH in the translocation of the enzymically active fragment A across lipid bilayers [17]. Using liposomes containing the toxin substrates NAD^+ and elongation factor 2, it was shown that only at low extravesicular pH and using intact diphtheria toxin could the enzymically active fragment A enter the liposomes and catalyse the ADP-ribosylation reaction. It is tempting to speculate that a similar translocation mechanism could operate for botulinum toxin. If, as has been suggested [5], BoNT/A initially enters the nerve by a process similar to receptor-mediated endocytosis, then the acidic environment which develops in the endosome [18] should induce the toxin to insert itself in the lipid bilayer. Whether or not the toxin or a toxin fragment subsequently enters the cytosol has yet to be determined. Recent studies may favour the translocation of the whole toxin since intact BoNT/A intracellularly injected into chromaffin cells strongly inhibits secretion [19]. Substantiation of such an internalisation mechanism would imply that the heavy subunit of BoNT/A plays a role in at least two stages in the action of the neurotoxin: firstly, in the binding of the toxin to the presynaptic nerve surface, most probably by an active site region located on the H_1 fragment [11], and secondly, in the translocation of the toxin or a fragment into the cytosol mediated by the H_2 component.

Clearly, in the light of the present findings and those with other bacterial toxins the interaction of botulinum neurotoxin with lipid bilayers at low pH is worthy of continued investigation.

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The N-terminal half of the heavy chain of botulinum type A neurotoxin forms channels in planar phospholipid bilayers

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The heavy chain of botulinum type A neurotoxin forms channels in planar phospholipid bilayer membranes. Channel activity is confined to the N-terminal half of this chain; the C-terminal half is inactive. Channel activity is stimulated by low pH (4.5–5.5) on the *cis* side (the side to which protein is added), neutral pH on the opposite *(trans)* side, and *cis* positive voltages. These findings are strikingly similar to those previously reported for analogous fragments of diphtheria and tetanus toxins.

Neurotoxin fragment; pH-dependent channel; Voltage gating; Lipid bilayer; Diphtheria toxin; Tetanus toxin; *(Clostridium botulinum)*

1. INTRODUCTION

The botulinum neurotoxins, which number among the most potent toxins known, cause a flaccid paralysis by blocking the release of acetylcholine from presynaptic cholinergic nerve terminals. The toxin is synthesized by the anaerobe *Clostridium botulinum* as a single polypeptide (M_r ~150000) which is split (termed nicking) into a heavy chain (M_r ~100000) and a light chain (M_r ~50000) by a protease endogenous to the bacteria or by mild trypsinization; the two chains are separated by reduction of the disulfide bond(s) which link(s) them (fig.1). The seven serologically distinct botulinum neurotoxin types recognized so far have a similar structure (see [1] and [2] for a general review of botulinum toxin). Interestingly, tetanus and diphtheria toxin share the same general macrostructure with botulinum neurotoxin [1].

Although a clear picture of the mechanism by which the toxin gains entry to the cytosol has yet to emerge, there may be an analogy with diphtheria toxin which is believed to employ cell-surface receptor binding, receptor-mediated endocytosis, and membrane translocation of its enzymatic light chain into the cytosol from an acidic vesicle compartment [3,4]. The exact molecular mechanism by which transmitter release is disabled also remains a mystery, although type D botulinum toxin has recently been shown to ADP-ribosylate a membrane protein of M_r ~21000 in bovine adrenal gland homogenate, suggesting that the mechanism is enzymatic [5].

It has previously been demonstrated that the heavy chains of botulinum type B neurotoxin,

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tetanus toxin and diphtheria toxin form large, voltage-dependent and pH-dependent ionic channels in planar lipid bilayers [6]. The channel-forming properties of the three toxins are remarkably alike, with channel activity maximal under the pH conditions which are likely to exist in an endocytic vesicle. This comparison makes compelling the suggestion that these pores formed by the heavy chain are involved in protein translocation of the light chain, and a possible role as 'tunnel proteins' has been suggested [6].

In this study we report that the heavy chain of botulinum type A neurotoxin, like that of type B, makes voltage-dependent and pH-dependent ionic channels in planar lipid bilayers. We further show that channel-forming activity is confined to the N-terminal half of the heavy chain; the C-terminal half of the heavy chain is devoid of channel-forming activity.

2. MATERIALS AND METHODS

2.1. *Neurotoxin and neurotoxin fragments preparation*

Botulinum type A neurotoxin was produced and purified as described [7], and its heavy and light chains were separated and purified chromatographically [8]. To cut the heavy chain, the whole neurotoxin (M_r ~145000) was digested with trypsin (EC 3.4.4.4) at a 10:1, w/w, ratio in 0.02 M Na_2HPO_4-NaH_2PO_4 buffer, pH 6.0, for 90 min at 30°C. The primary cleavage products were (i) light chain (M_r ~53000) linked to the N-terminal half of the heavy chain (M_r ~50000) by a disulfide bond (hence total M_r ~103000) and (ii) the C-terminal half (M_r ~47000) of the heavy chain; in fig.1 the two halves of the heavy chain are marked as H_2 and H_1. The two fragments (M_r ~103000 and ~47000) were purified by ion-exchange chromatography. The light chain (L) was then separated from the N-terminal half of the heavy chain (H_2), and the two were purified by ion-exchange chromatography. The two halves of the heavy chain were partially sequenced for characterization [9]. Details of fragmentation, purification and amino acid sequence determination will be published elsewhere (Sathyamoorthy, DasGupta, Niece and Foley, in preparation). The first 27 amino acid residues of the H_2 fragment (M_r ~50000) of the heavy chain [9] were identical to the N-terminal sequence of the intact heavy chain (M_r ~97000) [10]. This proved that (i) this fragment is the N-terminal half of the heavy chain, and (ii) the other half (M_r ~47000), whose 12 amino acid residues were sequenced [9], is apparently the C-terminal half of the heavy chain (H_1 fragment).

2.2. *Membrane formation and measurements*

Planar phospholipid bilayer membranes were formed at room temperature from the union of two lipid monolayers across a hole (0.1 to 0.2 mm diameter) in a Teflon partition [11] that had been pretreated with squalene; the partition separated two 1 ml compartments of a Teflon chamber containing buffered salt solutions, which were stirred independently by magnetic fleas. Monolayers were spread from 1% lipid solutions in hexane, and the solvent was allowed to evaporate before membrane formation. The lipid solutions consisted of either diphytanoylphosphatidylcholine (DPhPC) or a mixture of plant phosphatidylethanolamine (PE), plant phosphatidylcholine (PC), and bovine phosphatidylserine (PS) in the ratio PE/PC/PS of 2:2:1; all lipids were obtained from Avanti Polar Lipids, Birmingham, AL. The salt solutions contained 1 M KCl, 5 mM $CaCl_2$ and 0.1 mM EDTA. The *cis* solution (the one to which the toxin fragment was added) was buffered either at pH 4.7 with 5 mM dimethylglutaric acid (DMG) or at pH 5.5 with 5 mM 2-(*N*-morpholino)ethanesulfonic acid (Mes). The *trans* solution was buffered either at pH 7.4 with 5 mM Hepes or with the same buffer as in the *cis* solution. In the latter case, the pH of the *trans* solution was sometimes raised during the course of an experiment by stirring into it small aliquots of concentrated Hepes solution. After membrane formation, neurotoxin fragments were added from stock aqueous solutions to the *cis* compartment, to final concentrations of 0.1–1 μg/ml.

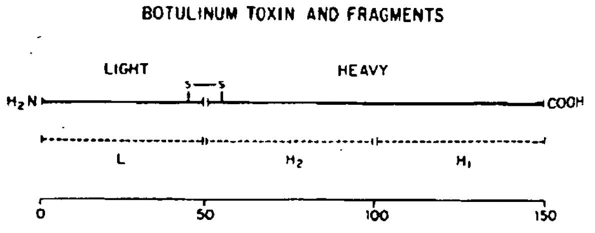

Fig.1. Botulinum neurotoxin and fragments. The scale is in units of kDa.

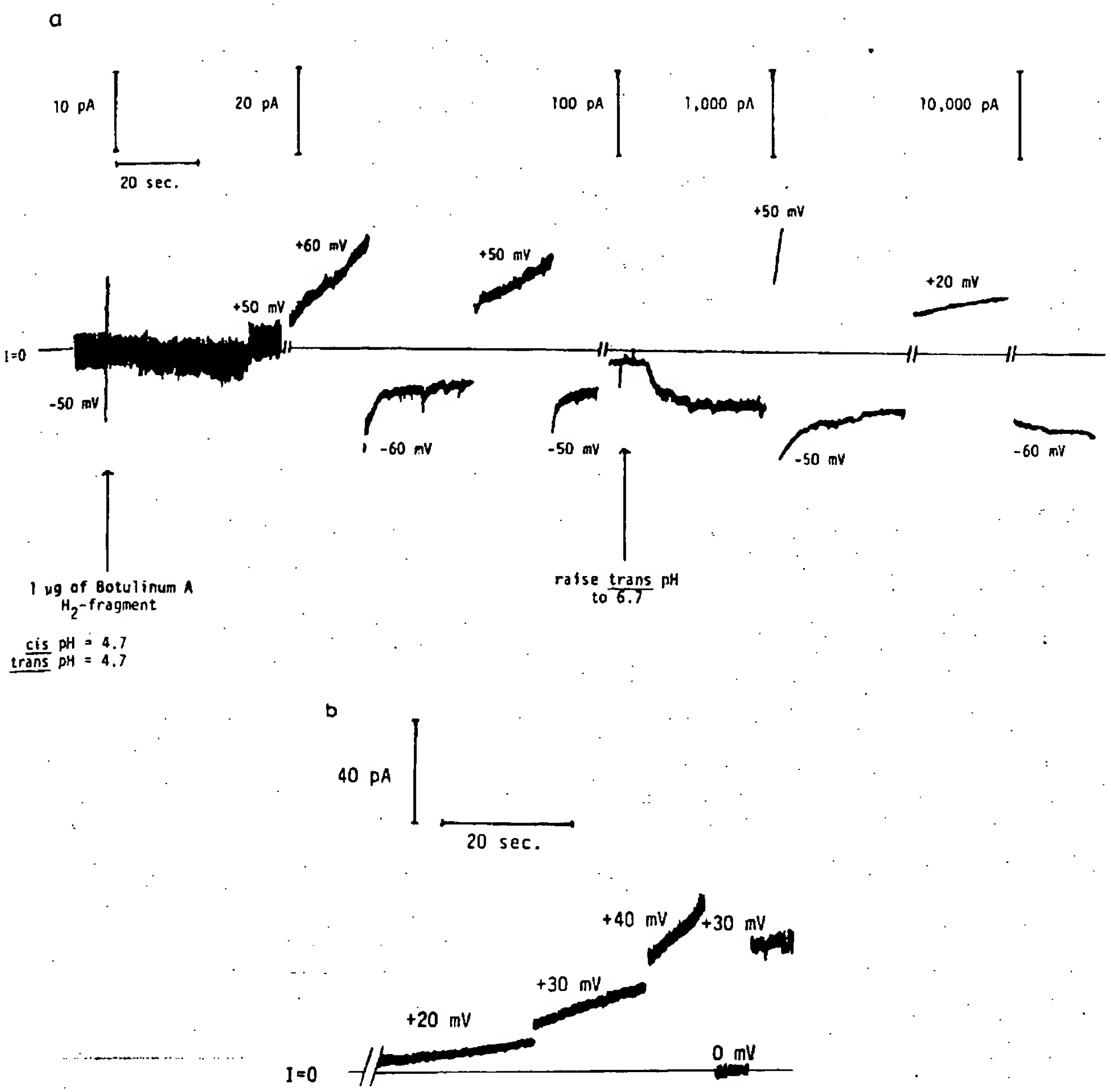

Fig.2. (a) Effect of raising *trans* pH on rates of channel opening and closing. Current traces are shown at different applied voltages. In the absence of toxin fragment, membrane current (and conductance) is virtually zero. Upon addition of ~1 µg of H_2 fragment to the *cis* compartment (volume 0.8 ml), the rate of channel opening (or closing) is seen as an increase (or decrease) in current with time. Rates of channel opening and closing at symmetric low pH (4.7) are compared to rates after raising the *trans* pH to 6.7. The rapid (5–10 s) increase in current (in the negative direction) seen immediately after raising the *trans* pH (see second arrow) is probably due to an increase in single-channel conductance. Further, the rate of channel opening at + 50 mV is increased by more than 50-fold over that before the elevation of the *trans* pH (note change in current scale). Solutions: 1 M KCl, 5 mM $CaCl_2$, 0.1 mM EDTA, in 5 mM DMG buffer, pH 4.7. *trans* pH was raised by addition of Hepes buffer. Lipid: PE/PC/PS (2:2:1 ratio). Voltage: All voltages refer to those of the *cis* side with respect to the *trans* side, which is defined as zero. (b) Effect of positive voltage on rate of channel opening. 10 min prior to the start of the record, H_2 fragment was added to the *cis* compartment to a concentration of ~0.2 µg/ml. Solutions: *cis*, 100 mM KCl, 5 mM Mes, pH 5.5; *trans*, 100 mM KCl, 5 mM Hepes, pH 7.4. Lipid: DPhPC.

Stock solutions of fragments were stored at 4°C at concentrations of ~150 μg/ml. Protein concentrations were estimations based on absorbance at 278 nm or intensity of Coomassie blue stained bands in polyacrylamide gels. Electrical measurements were made under voltage-clamp conditions using a single pair of Ag/AgCl electrodes, contacting the solutions via 3 M KCl agar bridges; current was monitored on a Narco physiograph chart recorder. The conductance (G) at any time is obtained from the relation $G = I/V$, where V is the voltage at which the membrane is clamped and I is the resulting current. The *trans* compartment was held at virtual ground; all voltages, therefore, refer to those of the *cis* compartment.

3. RESULTS

Several fragments of botulinum type A neurotoxin were examined for possible channel-forming activity: (i) the entire heavy chain; (ii) the N-terminal half of the heavy chain (H_2 fragment); (iii) the N-terminal half of the heavy chain linked via a disulfide bond to the light chain (H_2-L fragment); and (iv) the C-terminal half of the heavy chain (H_1 fragment) (see fig.1). The first three of these fragments listed were fairly similar in their channel-forming activity; differences which were evident include noise/fluctuation levels and potencies. In particular, experimental records with the heavy chain and the H_2-L fragment exhibited much more noise than those of the H_2 fragment. In addition, these fragments had to be present in ~5–10-fold higher concentrations than the H_2 fragment to achieve comparable conductances. When the C-terminal half of the heavy chain was examined under conditions which yielded maximal activity for the other fragments, no channel-forming activity was observed even when present at ~50-fold higher concentrations.

Addition of the H_2 fragment to one side of a planar lipid bilayer separating salt solutions at symmetric low pH (*cis*, 4.7; *trans*, 4.7) and subsequent clamping of the membrane potential to positive voltages results in steady rates of channel turn-on. (A typical record is shown in fig.2.) If the membrane is initially held at large negative potentials (< −50 mV), little or no activity is seen. Raising the *trans* pH causes a dramatic (>100-fold) increase in the rate of channel turn-on. This effect is apparent when one compares the rate of current increase at +50 mV at symmetric low pH to the rate after the *trans* pH has been raised to 6.7 (fig.2a, note the change in current scales). A second effect of raising the *trans* pH is a rapid (within stirring time) 3–5-fold increase in the steady-state conductance (fig.2a, second arrow). This is most likely due to an increase in the conductance of the single channels which comprise the macroscopic conductance, rather than an increase in the actual number of open channels. Consistent with this is a comparable rapid fall in conductance seen upon lowering of the *trans* pH from ~7.0 to ~5.0, presumably the result of a decrease in the single-channel size. No activity is exhibited by any of the active fragments when added to one side of a membrane separating solutions at symmetric neutral pH (or higher). If the *cis* pH is subsequently lowered to ≤5.5, however, full activity appears.

The effects of negative potentials on the channel kinetics are somewhat complicated (fig.2a). At symmetric low pH, large negative voltages (< −50 mV) result in a fast (2–5 s) phase of turn-off, followed by a much slower turn-off phase. This effect is also seen after the *trans* pH has been raised, but only initially; after ~1 min, potentials of −60 mV result in a turning-on of channels. It is likely that multiple open and closed states exist and that raising the *trans* pH drives channels into deeper open states. In addition to the gating of these channels by pH, there is also an effect of voltage on the rate of channel turn-on, with increasing positive voltages resulting in higher rates of turn-on (fig.2a,b).

4. DISCUSSION

It has previously been shown that botulinum neurotoxin types A, B, C_1, D and E [12,13], diphtheria toxin [14], and tetanus toxin [6,15] all form pH-dependent and voltage-dependent channels in planar lipid bilayer membranes. Furthermore, this channel-forming activity was found to be confined to the heavy chains of diphtheria toxin, tetanus toxin and botulinum type B neurotoxin [6,15,16]. In the present study, we have extended this finding to the heavy chain of botulinum type A neurotoxin. Moreover, we have shown that the channel-forming domain of the heavy chain is restricted to its N-terminal half (the H_2 fragment); the C-terminal half does not possess channel-forming activity. (Although our sample of the light chain was too dilute to adequately test for channel-forming activity, it is known from previous work [6] that the light chain of botulinum type B neurotoxin does not form channels.) This is precisely what was found earlier for diphtheria toxin and tetanus toxin [6,15], thereby strengthening the analogy among these three toxins.

Several striking similarities among the channel-forming properties of the botulinum, tetanus and diphtheria toxins are worthy of mention: (i) comparable fragments (the N-terminal half of the heavy chain) form channels; (ii) the channels manifest a similar voltage dependence, particularly the increase in channel activity with *cis* positive voltages; (iii) there is a requirement of low *cis* pH in channel formation; (iv) channel activity increases upon elevation of the *trans* pH; and (v) the single-channel conductance increases with the elevation of *trans* pH. With regard to this last point, although we have not yet investigated botulinum type A neurotoxin at the single-channel level, effects of pH on macroscopic records (see fig.2a) are consistent with those seen previously with botulinum type B neurotoxin (and with diphtheria toxin and tetanus toxin as well) [6,13].

A question which remains unanswered is the possible connection between channel formation by the heavy chain and protein translocation of the light chain. In addressing the general question of protein translocation one seeks to find the means by which nature solves the problem of overcoming the significant energy barrier which polar, hydrophilic regions must overcome in their journey across the low dielectric medium of the plasma (or vesicle) membrane. In the light of this, and the similarity of conditions required for channel formation in bilayers and intoxication in cells, it has been suggested that the aqueous pores formed by the heavy chains of these toxins may accommodate the passage of the light chains in an unfolded conformation [6,16].

The genes for both tetanus toxin [17] and diphtheria toxin [18,19] have been cloned and sequenced and the primary structures of these toxin proteins thereby deduced. In recent years, the use of molecular cloning techniques in the study of ionic channels has provided a unique probe into the molecular mechanisms which underlie channel-forming activity. We hope to eventually bring this technique to bear on the channels formed by the botulinum neurotoxins.

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ANNEX 8

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Purification and Properties of a D-Galactose/*N*-Acetyl-D-galactosamine-Specific Lectin from *Erythrina cristagalli*

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The lectin from the seeds of *Erythrina cristagalli* has been isolated in high yield (75%) and homogeneous form by affinity chromatography on a column of D-galactose-derivatised Sepharose. It is a glycoprotein with a molecular weight of 56800 ± 900 and $s_{20,w} = 3.9$ S, composed of two subunits (apparent molecular weights of 28000 and 26000 respectively) both of which are glycosylated. The total carbohydrate content is 4.5% and it is comprised of mannose, *N*-acetylglucosamine, fucose and xylose in amounts corresponding to 7, 4, 2 and 2 mol/56800 Da respectively. The amino acid composition of the lectin is characterised by a high content of acidic and hydroxy amino acids, low content of methionine and absence of cysteine. Valine is the only N-terminal amino acid detected. The lectin is a metalloprotein in that it contains 0.093% Mn and 0.13% Ca (1 mol and 1.9 mol/56800 Da respectively), both of which are tightly bound to the protein.

E. cristagalli lectin agglutinates untreated human erythrocytes of all blood types, as well as rabbit erythrocytes, at a concentration of 5–10 µg/ml. It is mitogenic for human peripheral blood T lymphocytes at an optimal concentration of about 100 µg/ml, but is not mitogenic for mouse thymocytes or splenocytes.

D-Galactose and various D-galactosides inhibit the hemagglutinating activity of the lectin. *N*-Acetyllactosamine is most potent, completely inhibiting four agglutinating units of the lectin at 0.4 mM concentration. Lactose, *N*-acetyl-D-galactosamine and D-galactose are 5, 16 and 35 times less active respectively. Lactose specifically perturbs the ultraviolet spectrum of the lectin in the aromatic region. The difference spectrum obtained upon binding of the disaccharide to the lectin shows maxima at 291 nm and 282–284 nm, indicating a change in the environment of tryptophan residues of the protein upon binding of sugar.

During the last decade there has been a remarkable increase of interest in lectins. Close to 100 lectins have been purified and characterised, and some of them were found to possess unusual structural features (for recent reviews see [1,2]). The sugar specificity of many lectins, and their interactions with different cells, have been investigated. As a result of these studies it became clear that lectins with identical specificity with respect to monosaccharides differ in their interactions with oligosaccharide moieties of glycoconjugates, and therefore also with cells. For example, of all known galactose[1]-specific lectins, only peanut agglutinin distinguishes between mature and immature human and murine thymocytes, while among *N*-acetylgalactosamine-specific lectins only soybean agglutinin interacts selectively with murine B splenocytes and not with T cells [3]. It is therefore important to isolate as many lectins as possible and to study their sugar specificity in detail.

In this paper we describe the isolation of a galactose/*N*-acetylgalactosamine-specific lectin from the seeds of *Erythrina cristagalli* by affinity chromatography on galactose-derivatised Sepharose. The lectin has been studied with respect to its structure, composition, biological activities and sugar specificity. Its properties are compared with those of the recently purified lectins from *Erythrina indica* [4] and *Erythrina corallodendrum* [5].

MATERIALS AND METHODS

Materials

Divinylsulphone was a product of Polyscience (Warrington, PA), Sepharose 6B was from Pharmacia (Uppsala) and concanavalin A–Sepharose was from Miles-Yeda (Rehovot). Neuraminidase from *Vibrio cholerae* (1 U/ml) was purchased from Behringwerke (Marburg): 1 unit is defined as the amount of enzyme that liberates 1 µmol sialic acid from human α_1 acid glycoprotein in 1 min at 37 °C and pH 5.5. Trypsin (2 × crystallised), human transferrin and bovine serum albumin were from Sigma (St Louis), ovalbumin and lysozyme from Worthington (New Jersey), and fetuin from Gibco (Grand Island, NY). *N*-Acetyllactosamine was a synthetic product [6] kindly supplied by Dr A. Veyrières (Université de Paris-Sud). All other sugars were commercial products of highest purity available and all other reagents were of analytical grade. Soybean agglutinin was prepared as described [7] except that acid-treated Sepharose [8] was used as adsorbent for affinity chromatography. *Erythrina cristagalli* seeds were collected by one of us (J. L. I.) in Uruguay. Slightly outdated human blood was obtained from Kaplan Hospital, Rehovot, and rabbit, sheep and mouse blood was drawn from animals supplied by the Animal Breeding Centre at the Weizmann Institute. Asialofetuin was prepared by hydrolysis of fetuin (20 mg/ml, 0.1 M HCl, 80 °C for 1 h) and dialysis of the hydrolysate against phosphate-buffered saline, P_i/NaCl (0.05 M potassium/sodium phosphate buffer, pH 7.2, in 0.9%

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[1] All sugars were of the D-configuration unless otherwise stated.

NaCl). To obtain neuraminidase-treated cells a 20% suspension of erythrocytes in P_i/NaCl was treated with 5 mU enzyme/ml for 1 h at 37 °C and the cells washed three or four times in P_i/NaCl. Trypsinization of erythrocytes was carried out on a 4% cell suspension in P_i/NaCl using 100 µg enzyme/ml, and the cells were washed as above.

Preparation of Affinity Column

Galactose-derivatised Sepharose 6B was prepared by the divinylsulphone method of Porath and Ersson [9]. Packed Sepharose 6B (100 g wet weight) was suspended in 100 ml 0.5 M sodium carbonate buffer, pH 11, and 10 ml divinylsulphone was added. The suspension was kept at room temperature for 70 min with slow stirring and the activated gel was thoroughly washed on a glass filter with distilled water. It was then suspended in 100 ml of a 10% solution (w/v) of galactose in the carbonate buffer and left overnight in the cold room (4 – 6 °C). The resulting product was washed on a glass filter with 1 l carbonate buffer followed by 2 l water and suspended in P_i/NaCl.

Protein Estimation

The method of Lowry et al. [10] was routinely used with bovine serum albumin as standard. For the purified lectin, concentration was estimated from absorption measurements at 280 nm, using the factor $A^{1\%}_{1\,\mathrm{cm}} = 12.4$. This value was obtained by determining the protein concentration by the method of Lowry et al. [10] of a solution of purified lyophilised lectin of known absorbance.

Carbohydrate Determinations

Total neutral carbohydrate content was determined by the phenol/sulphuric acid method [11] using mannose as reference sugar. Individual monosaccharides were determined by gas chromatography as trimethylsilyl derivatives after methanolysis of the lectin in the presence of mannitol as internal standard. A column of 3% SE-30 in the temperature range 140 – 200 °C at 0.5 °/min was used [12].

Amino Acid Analysis

Amino acid composition was determined on a Dionex D500 amino acid analyser. Hydrolysis was in sealed tubes under nitrogen at 110 °C with 6 M HCl for 22 h, 48 h and 72 h. Values for serine and threonine were obtained by extrapolation to zero-time hydrolysis and the maximal values were taken for the remaining amino acids. Methionine and cysteine were determined as methionine sulphone and cysteic acid, respectively, after oxidation of the protein with performic acid [13]. For estimation of tryptophan, hydrolysis was carried out in 4 M methanesulphonic acid [14]. N-terminal amino acids were determined by Edman degradation on a Beckman model C sequencer with quadrol [*N,N,N′,N′*-tetrakis(2-hydroxypropyl)ethylenediamine] buffer. Identification of amino acid phenylthiohydantoins was done by high-performance liquid chromatography on a Zorbax 5-µm (Dupont) column.

Metal Content

Analysis of metals was done by atomic absorption on a Perkin-Elmer 306 Spectrophotometer. Salt solutions were prepared with double-distilled water and demetallised on a column of Chelex 100 (Bio-Rad). The lectin solution used for metal determination was dialysed at 4 °C with four changes of each external solution against (a) metal-free saline, (b) 0.1 M EDTA followed by metal-free saline, or (c) 1 M acetic acid, followed by metal-free saline. Any precipitate that formed during dialysis was removed by centrifugation.

Gel Electrophoresis

Discontinuous polyacrylamide gel electrophoresis at pH 8.9 was performed according to Davis [15]. Electrophoresis in the presence of sodium dodecylsulphate was carried out on 10% gels in the discontinuous buffer system of Laemmli [16]. For the determination of the apparent molecular weight of the subunits the gels were calibrated with transferrin (M_r 76000), bovine serum albumin (M_r 68000), ovalbumin (M_r 46000), soybean agglutinin (subunit M_r 30000) and lysozyme (M_r 14000). The gels were stained for protein with Coomassie brilliant blue R 250 and for carbohydrates with the periodate/Schiff reagent [17].

Ultracentrifugal Studies

The sedimentation coefficient was determined in a Spinco model E analytical centrifuge at 52000 rev./min and 20 °C using a 0.57 mg/ml solution of the lectin in P_i/NaCl. The molecular weight was calculated from sedimentation equilibrium data according to Yphantis [18], obtained at 17000 rev./min and a protein concentration of 0.35 mg/ml. The partial specific volume was calculated [19, 20] from the amino acid and sugar composition as 0.724 ml/g.

Affinity Chromatography on Concanavalin-A – Sepharose

Purified *E. cristagalli* lectin (5 mg dissolved in 1 ml P_i/NaCl) was applied to a column (1 × 4 cm) of concanavalin-A – Sepharose, and left in contact with the adsorbent for 15 min at 4 °C. The column was then washed with 10 ml P_i/NaCl and the adsorbed lectin eluted with 0.2 M methyl α-mannoside. Elution was followed by monitoring the absorbance of the effluent at 280 nm.

Hemagglutinating Activity

The hemagglutinating activity of the lectin was assayed by the serial dilution method on microtiter plates, using 50 µl lectin solution and 50 µl of a 4% suspension of erythrocytes. A unit of activity is defined as the lowest concentration of lectin giving visible agglutination. The inhibitory activity of sugars was measured by mixing serial dilutions of the inhibitor with four hemagglutinating units of the lectin before addition of erythrocytes and determining the lowest concentration giving full inhibition of agglutination.

Mitogenic Activity

Human lymphocytes were isolated from freshly drawn peripheral blood by centrifugation on Ficoll-Hypaque [21]. T cells were separated from B cells by rosetting out with sheep erythrocytes [22]. Preparation of mouse thymocytes and splenocytes, treatment of the cells with neuraminidase, culture conditions and measurement of the stimulation of DNA synthesis, were carried out as previously described [23].

Table 1. *Purification of* E. cristagalli *lectin*
The crude extract was obtained from 100 g defatted meal

Step	Volume	Total protein	Specific activity	Total activity	Recovery	Purification
	ml	mg	units/mg	units	%	-fold
Crude extract	800	19000	3	51200	100	1
$(NH_4)_2SO_4$ precipitation						
0–30% fraction	200	2200	0.7	1540	3	
30–60% fraction	200	4620	9	41580	82	3
60–100% fraction	330	5280	0.5	2640	5	
Affinity chromatography	150	180	210	37800	74	70

Purification of E. cristagalli *Lectin*

Unless otherwise stated, all operations were carried out in the cold room. Finely ground seeds were defatted by extraction with petroleum ether and air dried at room temperature. The defatted meal (30 g) was extracted with 300 ml P_i/NaCl for 1 h with stirring, the extract was filtered through cheese-cloth and clarified in a Sorvall centrifuge at 13000 rev./min for 10 min. Ammonium sulphate (17.6 g/100 ml) was added to the supernatant with stirring, and the precipitate was removed by centrifugation as above; more ammonium sulphate (19.8 g/100 ml) was added and the mixture kept overnight. The precipitate was collected by centrifugation, suspended in distilled water (80–90 ml) and dialysed extensively, first against distilled water (2 × 5 l) and finally against P_i/NaCl. Any precipitate that formed during dialysis was removed by centrifugation and the clear supernatant was applied to a column (2 × 15 cm) of galactose-derivatised Sepharose, equilibrated with P_i/NaCl. The column was washed with the same buffer until the absorbance at 280 nm of the effluent was ≤ 0.05 and the bound lectin was eluted with 0.2 M galactose. Elution was followed by monitoring the absorbance at 280 nm; the fractions containing protein were collected, dialysed extensively against distilled water and lyophilised.

RESULTS AND DISCUSSION

The presence of hemagglutinating activity in extracts from seeds of different species of *Erythrina* has been known for a long time [24], but only recently have two of the lectins, namely from *Erythrina indica* [4] and from *Erythrina corallodendrum* [5] been purified and characterized.

The *Erythrina cristagalli* lectin described in this paper is eluted from a column of Sepharose-bound galactose as a sharp peak (Fig. 1); neither Sepharose alone, nor acid-treated Sepharose [8] could be used as affinity support, since the lectin did not bind at all to the former and only weakly to the latter. About 50 mg lectin (yield 75%) is obtained from 30 g defatted meal, with a specific hemagglutinating activity of approximately 200 units/mg when determined with untreated human O-type erythrocytes. The purification procedure is summarised in Table 1. As can be seen, the lectin comprises about 1.2% of the soluble proteins of the seeds.

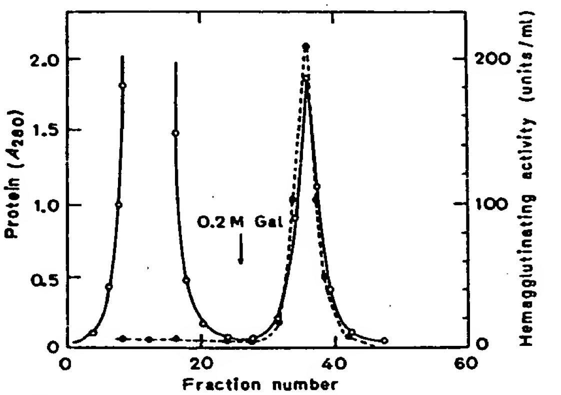

Fig. 1. *Affinity chromatography of* E. cristagalli *lectin on a column of galactose-derivatised Sepharose 6B.* The dialysed ammonium sulphate precipitate obtained from 30 g defatted meal was applied to a 2 × 15-cm column equilibrated with P_i/NaCl. Elution was with 0.6 M galactose. (○——○) A_{280}; (●---●) hemagglutinating activity (units/ml)

The purified lectin is homogenous by polyacrylamide gel electrophoresis at pH 8.9. It gives a single symmetrical peak in sedimentation velocity and equilibrium experiments, with $s_{20,w} = 3.9$ S and M_r of 56800. Upon polyacrylamide gel electrophoresis in the presence of sodium dodecylsulphate, two closely migrating bands are obtained, with apparent molecular weights of 28000 and 26000 respectively (Fig. 2). These values are very close to those reported for the *E. indica* lectin ($s_{20,w} = 4.0$ S, molecular weight 66200, and two subunits with molecular weights of 34000 and 30000 [4]). In contrast, the lectin from *E. corallodendrum* has a molecular weight of 110000–120000 [5].

The two bands obtained by polyacrylamide gel electrophoresis of the *E. cristagalli* lectin stained for protein as well as for carbohydrate, indicating that the lectin is composed of two subunits and that both are glycosylated. Homogeneity, as well as the glycoprotein nature of the lectin, was confirmed by chromatography on concanavalin A–Sepharose. The lectin is quantitatively adsorbed to the column and more than 80% of the adsorbed protein is eluted as a sharp peak with 0.2 M methyl α-mannoside (Fig. 3). The combined fractions of the peak contain essentially the total hemagglutinating activity applied to the column. The neutral carbohydrate content of the lectin, as determined by the phenol/sulphuric acid method, is 2.8%. Based on gas chromatography analysis (Table 2), the lectin contains mannose, *N*-acetylglucosamine, fucose and xylose in a molar ratio of 3.5 : 2 : 1 : 1, in amounts corresponding to 7, 4, 2 and 2 mol/56800 Da respectively; the total carbohydrate content was calculated to be 4.5%. A strikingly similar carbohydrate composition has been reported for bromelain, the proteolytic enzyme from pineapple stem [25]. It seems likely that, in analogy to other glycoproteins [26,27], the carbohydrate is linked to the protein via GlcNAc-GlcNAc-

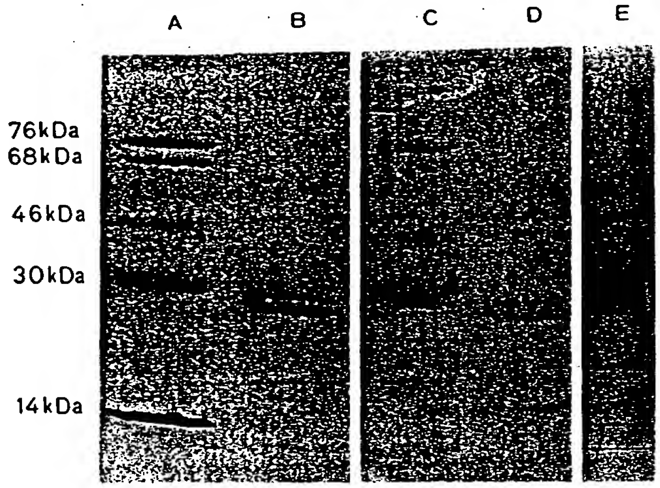

Fig. 2. *Polyacrylamide gel electrophoresis (10%) of purified* E. cristagalli *lectin.* Tracks A–D: in the presence of sodium dodecylsulphate; track E: pH 8.9 without sodium dodecylsulphate. Tracks C and D were stained for carbohydrates with the periodate/Schiff reagent. The markers used (tracks A and C) were transferrin (M_r 76000); bovine serum albumin (M_r 68000); ovalbumin (M_r 46000), soybean agglutinin (subunit M_r 30000); lysozyme (M_r 14000)

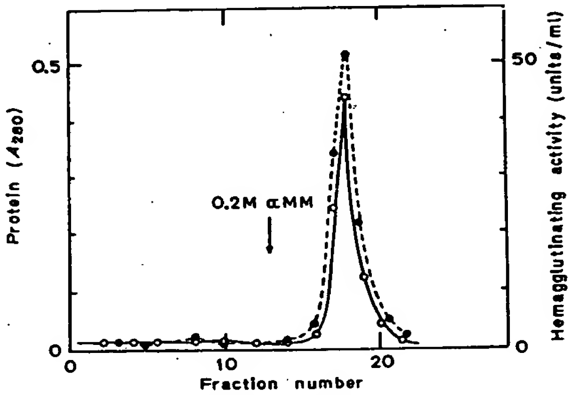

Fig. 3. *Affinity chromatography of purified* E. cristagalli *lectin on a column of concanavalin-A – Sepharose.* 5 mg lectin in 1 ml P_i/NaCl was applied to a 1×4-cm column. Elution was with 0.2 M methyl α-mannoside (α MM). (○—○) A_{280}; (●---●) hemagglutinating activity (units/ml)

Table 2. *Carbohydrate composition of* E. cristagalli *lectin*
The monosaccharides were determined by gas chromatography as trimethylsilyl derivatives. The value for *N*-acetylglucosamine is based on the glucosamine content. Results are based on a molecular weight of 56800

Monosaccharide	Composition
	mol/mol
Fucose	1.9
Xylose	2.0
Mannose	7.1
N-Acetylglucosamine	3.8

Table 3. *Amino acid composition of* E. cristagalli *lectin*
Results are based on a molecular weight of 56800 with 4.5% carbohydrate

Amino acid	Composition	
	residues/mol	mol/100 mol
Asp	62	11.5
Thr	43	8.0
Ser	47	8.9
Glu	55	10.2
Pro	39	7.2
Gly	39	7.2
Ala	40	7.4
Half-Cys	0	0
Val	42	7.8
Met	6	1.1
Ile	30	5.6
Leu	37	6.9
Tyr	20	3.7
Phe	28	5.2
His	8	1.5
Lys	20	3.7
Arg	11	2.0
Trp	11	2.0

Table 4. *Agglutination of erythrocytes from various species by* E. cristagalli *lectin*

Origin of erythrocytes	Minimal hemagglutinating dose		
	untreated	trypsinised	neuraminidase-treated
	μg/ml		
Human A type	10		
Human B type	10		
Human O type	5	0.6	1.2
Rabbit	10	0.16	n.d.
Mouse	*	*	30
Sheep	*	*	4

* No agglutination at 1 mg lectin/ml.
n.d. = not determined.

Asn. Therefore, there is probably one carbohydrate chain per subunit, in which the mannose residues are attached to the distal *N*-acetylglucosamine in a branched structure.

The amino acid composition of *E. cristagalli* lectin is shown in Table 3. In common with many lectins, it is devoid of cysteine and has a low content of methionine, but is rich in acidic and hydroxy amino acids. Valine is the only N-terminal amino acid detected. The lectin contains 0.093% Mn (1 mol/56800 Da) and 0.13% Ca (1.9 mol/56800 Da). Exhaustive dialysis against 0.1 M EDTA or 1 M acetic acid does not decrease the metal content and hemagglutinating activity of the preparation, although the latter treatment has been shown to lead to the demetallization and loss of biological activity of many lectins [28, 29]. When hemagglutination is carried out in the presence of EDTA at a final concentration of 0.05 M, no effect on the agglutinating activity of the lectin is observed. With the galactose/*N*-acetylgalactosamine-specific lectin from *Sophora japonica*, EDTA at a concentration as low as 0.23 mM completely inhibited four agglutinating doses of the lectin [30]. From the above results, no conclusion can be drawn concerning the importance of the metals for the biological activity of the *E. cristagalli* lectin. It appears, however, that they are tightly bound to the protein.

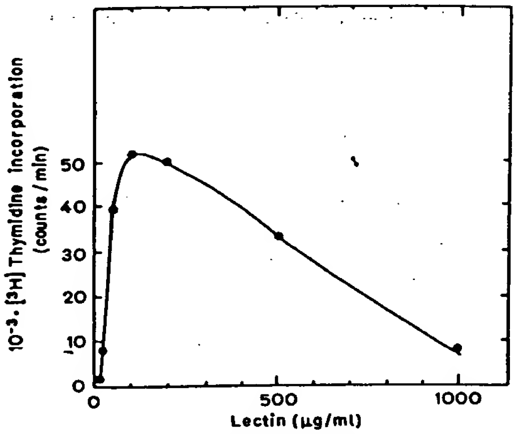

Fig. 4. *Mitogenic stimulation of human peripheral blood lymphocytes by purified* E. cristagalli *lectin.* Cells (2×10^5) were cultured for 48 h with 0.2 ml RPMI 1640 in the presence of varying amounts of lectin. [^{3}H]-Thymidine was added 6 h before harvesting

Table 5. *Mitogenic activity of* E. cristagalli *lectin tested with human peripheral blood T and B lymphocytes*
Assay conditions as described in legend to Fig. 4. Results are expressed as the stimulation index, i.e. ratio of thymidine incorporated in the presence and absence of lectin

Lymphocytes	Stimulation index with the following concn (μg/ml) lectin in assay					
	25	50	100	250	500	1000
Total peripheral blood	13	41	29	6	2	2
T cells	20	67	52	10	3	2
B cells	4	6	9	3	3	1

Table 6. *Inhibition by various sugars of the hemagglutinating activity of* E. cristagalli *lectin with human O erythrocytes*
Other sugars tested, i.e. glucose, *N*-acetylglucosamine, mannose, methyl α-mannoside, xylose, L-fucose, L-arabinose and L-rhamnose were non-inhibitory at concentrations of 0.1 M. The minimal inhibitory concentrations is that required to inhibit completely 4 units of the lectin. The values given are the average of four determinations, starting with different concentrations of the inhibitors. The results are accurate within ± 30%

Inhibitor	Minimal inhibitory concentration
	mM
N-Acetyllactosamine	0.4
Lactose	2.0
p-Nitrophenyl β-galactoside	2.0
p-Nitrophenyl α-galactoside	4.0
N-Acetylgalactosamine	6.5
Methyl α-galactoside	4.8
Methyl β-galactoside	13.5
Galactose	13.5
D-Fucose	18.0
Raffinose	7.2
Galactosamine	20.0
	mg/ml
Asialofetuin	1.56

The lectin agglutinates untreated human erythrocytes of all blood types (with a slight preference for O-type cells), as well as rabbit erythrocytes, at a concentration of 5–10 μg/ml (Table 4). With trypsinised cells about 20-fold lower concentrations of the lectin are required to cause agglutination. Treatment of human erythrocytes with neuraminidase decreases the minimal agglutinating concentration of the lectin by a factor of four. On the other hand, this enzyme has a very strong effect on the susceptibility to agglutination of sheep and mouse erythrocytes: microgram quantities of the lectin are sufficient to agglutinate neuraminidase-treated cells from both animals, while untreated and trypsinised cells are not agglutinated even at 1 mg lectin/ml.

The lectin is mitogenic for untreated (Fig. 4) and desialylated (results not shown) human peripheral blood lymphocytes, at an optimal concentration of about 100 μg/ml for both types of cell. As can be seen from Table 5, the mitogenic activity is directed specifically towards T lymphocytes. The lectin, however, does not stimulate untreated or neuraminidase-treated mouse thymocytes or splenocytes. No information on the mitogenic activity of the *E. indica* lectin is available, but the *E. corallodendrum* lectin is active only with neuraminidase-treated cells.

Of the various carbohydrates tested for inhibition of hemagglutination by *E. cristagalli* lectin, glucose, *N*-acetylglucosamine, mannose, methyl α-mannoside, xylose, L-fucose, L-arabinose and L-rhamnose have no effect up to a concentration of 0.1 M, whereas galactose and all saccharides containing this sugar at the non-reducing end, exhibit various degrees of inhibitory activity (Table 6). *N*-Acetyllactosamine [Gal-β(1-4)GlcNAc] is most potent, completely inhibiting four units of the lectin at 0.4 mM concentration. Lactose and galactose are 5 and 35 times less active respectively. The inhibitory activity of *N*-acetylgalactosamine is between that of lactose and that of galactose. The inhibition studies thus show that the *E. cristagalli* lectin recognises the configuration at the C-4 of the galactopyranoside ring. Replacement of the hydroxyl group at C-2 with an acetamido group increases the inhibitory activity of the sugar; the presence of an amino group greatly decreases this activity. A hydroxymethyl (or methyl) group at C-5 is also important, since L-arabinose is not an inhibitor. The fact that *N*-acetyllactosamine is the best inhibitor and that this disaccharide is five times more active than lactose, strongly suggests that the lectin possesses an extended binding site and that the 2-acetamido group on the penultimate sugar is important for recognition by the lectin.

Although the lectin possesses an extended binding site, galactosides are only slightly better inhibitors than is free galactose. The *p*-nitrophenyl derivatives are not significantly different from methyl galactosides. However, while methyl α-galactoside is a stronger inhibitor than its β anomer, with *p*-nitrophenyl derivatives the β anomer is more potent than the α anomer. Similar behaviour has been found with other galactose/*N*-acetylgalactosamine-specific lectins, e.g. that from *S. japonica* [30], *Wistaria floribunda* [31], and *Psophocarpus tetragonolobus* [32].

Lactose perturbs the ultraviolet spectrum of the lectin in the aromatic region (Fig. 5). The effect is specific, since no change in the spectrum is obtained upon treatment of the lectin with glucose. The difference spectrum obtained upon binding of the disaccharide shows maxima at 291 nm and 282–284 nm, indicating a change in the environment of tryptophan residues upon sugar binding [33].

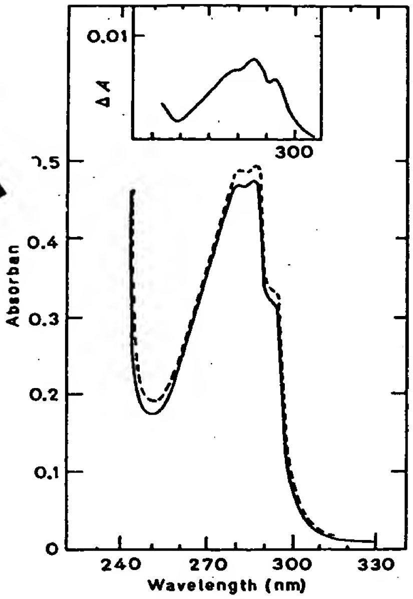

Fig. 5. *Ultraviolet spectrum of* E. cristagalli *lectin (0.4 mg/ml) in the presence of 25 mM lactose (---) and in its absence (——).* Inset: difference spectrum

A comparison of the sugar specificities of the known *Erythrina* lectins reveals many similarities, in particular between those of *E. corallodendrum* and *E. cristagalli* lectins. The lectin from *E. indica* differs from the two other lectins only in that *N*-acetylgalactosamine appears to be four times more efficient an inhibitor than lactose.

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ANNEX 9

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Expression of *Erythrina corallodendron* lectin in *Escherichia coli*

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The cDNA of the *Erythrina corallodendron* lectin (*ECor*L) has been expressed in *Escherichia coli*. For this purpose, an *Nco*I site was inserted into the cDNA coding for the lectin precursor [Arango, R., Rozenblatt, S. & Sharon, N. (1990) *FEBS Lett. 264*, 109 – 112] immediately before the codon GTG (103 – 105) which codes for the N-terminal valine of the mature lectin. This introduced an ATG codon for a methionine preceding the valine. The mutated cDNA was ligated into pUC-8, then subcloned into the expression vector pET-3d, which carries a strong promoter derived from gene 10 of the phage T7. The recombinant plasmid was introduced into the *E. coli* lysogenic strain BL21(DE3). Recombinant *ECor*L was expressed by growing the bacteria in the presence of isopropyl β-D-thiogalactopyranoside. Most of the recombinant lectin was found in an insoluble aggregated form as inclusion bodies and only a small part was in the culture medium in a soluble active form. Functional recombinant lectin was recovered from the inclusion bodies by solubilization with 6 M urea in cyclohexylaminopropane sulfonate pH 10.5, renaturation by 10-fold dilution in the same buffer and further adjustment of the pH to 8.0. The recombinant lectin, obtained at a yield of 4 – 7 mg/l culture, had, by gel filtration, a slightly lower molecular mass (56 kDa) than the native lectin, and was devoid of covalently linked carbohydrate; it was, however, essentially indistinguishable from native *ECor*L by other criteria, including its dimeric structure, Western blot analysis with anti-*ECor*L polyclonal and monoclonal antibodies, and Ouchterlony double-diffusion analysis with polyclonal antibodies, as well as hemagglutinating activity and specificity for mono- or disaccharides.

Lectins are carbohydrate-binding proteins present in a variety of organisms ranging from bacteria to higher vertebrates [1, 2]. The most thoroughly studied lectins are those extracted from plants, especially from the seeds of the leguminosae [3, 4]. They serve as invaluable carbohydrate-specific tools in many areas of biological and medical research, and are also used clinically. Legume lectins comprise a large group of proteins that share extensive similarities in their primary amino acid sequences, and possess similar secondary and tertiary structures [4 – 6]. Despite these similarities, they display a wide range of carbohydrate specificities. The structural basis of these specificities is most likely due to differences in the architecture of the variable parts of their binding sites [7].

Erythrina is a family of over 100 species of deciduous leguminous trees and shrubs found in the tropics and subtropics. Since 1980, lectins from over 20 species of this family have been isolated in different laboratories, ten of these by us [3, 8]. All *Erythrina* lectins are composed of two identical, or nearly identical, subunits with molecular masses close to 30 kDa. They are glycoproteins containing 3 – 9% carbohydrate and are specific for galactose and *N*-acetylgalactosamine, with a preference for *N*-acetyllactosamine which binds 10 – 30 times better than galactose [8 – 10]. Recently, the three-dimensional structure of the complex of *Erythrina corallodendron* lectin (*ECor*L) with lactose has been solved at 0.2-nm resolution [7].

We had previously cloned and sequenced a 1017-bp cDNA fragment containing the entire coding region of *ECor*L [11]. This fragment encodes a polypeptide of 281 amino acids, consisting of a leader sequence of 26 amino acids and a mature lectin of 255 amino acids. We now report the expression and recovery of functional recombinant *ECor*L (r*ECor*L) in *Escherichia coli*. This was accomplished by introducing the region coding for the mature *ECor*L into an expression vector based on the T7 promoter. The availability of this expression system will enable us to perform structure/function relationship studies of the lectin's binding site by site-directed mutagenesis, and also to attempt to modify the specificity of the lectin.

MATERIALS AND METHODS

Materials

Restriction enzymes and DNA-modifying enzymes were obtained from New England Biolabs and United States Biochemical Corporation (Cleveland, OH). DNA sequencing reagents were also obtained from US Biochemical and *Taq* DNA polymerase was obtained from New England Biolabs. *Bam*HI linkers were from Pharmacia; oligonucleotides were from the Chemical Services of the Weizmann Institute of Science; pET-3d was from Novagen (Madison, WI). Native *ECor*L was isolated by affinity chromatography on immobilized galactose

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Abbreviations. Caps, 3-(cyclohexylamino)-1-propanesulfonic acid; *ECor*L, *Erythrina corallodendron* lectin; r*ECor*L, recombinant *ECor*L; IPTG, isopropyl β-D-thiogalactopyranoside; PCR, polymerase chain reaction.

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[8, 12] and further purified by affinity chromatography on immobilized concanavalin A as described for *Erythrina cristagalli* lectin [12]. All other chemicals were from commercial sources, of the highest purity available.

Plasmids and fragments

Plasmid DNAs were prepared by the alkali/SDS-lysis procedure and purified with Qiagen-tip20 columns (Diagen GmbH, Düsseldorf) according to the manufacturer's protocols. DNA fragments were purified by extraction from a low-melting agarose gel using standard procedures [13]. Plasmid DNAs were introduced into various strains of *E. coli* by calcium-mediated transformation [13].

DNA manipulations for construction of expression vectors

Digestions with restriction enzymes were performed using buffers and conditions supplied by the manufacturers. When complete digestion was necessary, the reaction mixtures were left overnight. Ligations were done in a reaction mixture containing ligation buffer (50 mM Tris/HCl pH 7.6, 10 mM $MgCl_2$ and 1 mM ATP), 5 units T4 DNA ligase and the DNAs to be ligated at appropriate concentrations [13]; incubation was overnight at 15°C. DNA sequencing was carried out by the dideoxy-chain-termination method [14], using Sequenase version 2.0 kit according to the protocol provided by US Biochemical Corp.

Polymerase chain reaction

The amplification of the *ECor*L mutated fragment was performed in a 100-µl reaction volume containing polymerase chain reaction (PCR) buffer (10 mM Tris/HCl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin), dNTPs (20 µM each), 50 pmol 3′ and 5′ primers, 200 ng plEcl-C (a plasmid containing the *ECor*L cDNA) [11], 2.5 units *Thermus aquaticus* DNA polymerase and two drops of mineral oil. The samples were placed in a thermal controller (MJ Research) programmed for a temperature cycle of 95°C (1 min), 37°C (2 min) and 72°C (2 min). This cycle was repeated 30 times with a 7-min extension at 72°C following the last cycle. The final reaction products were resolved in a 1% agarose gel. The amplified fragment was prepared for subcloning by a fill-in reaction with T4 DNA polymerase.

Expression of *ECor*L

E. coli BL21(DE3), a lysogen containing a single gene of the T7 RNA polymerase under the control of the inducible lacuv5 promotor, was used for expression of *ECor*L [15]. The bacteria with pET-*ECor*L or control pET-3d were grown at 37°C, with shaking, in 10 ml NZYM medium (10 g type A hydrolysate of casein, 5 g NaCl, 5 g yeast extract and 2 g $MgSO_4 \cdot 7\ H_2O$) [13] containing 0.4% glucose and 100 µg/ml ampicillin until mid-log phase (A_{600} of 0.6), then induced by the addition of isopropyl β-D-thiogalactopyranoside (IPTG) to 0.4 mM and incubation was continued at 25°C or 37°C. Aliquots (1 ml) of bacterial culture were taken after predetermined periods of induction (up to 24 h). The cells were collected by centrifugation and suspended in 80 µl double distilled water and 20 µl SDS/PAGE sample buffer (0.31 M Tris pH 6.8, 10% SDS, 50% glycerol and 0.005% bromophenol blue). Detection of *ECor*L in these bacterial lysates after SDS/PAGE was done by staining with Coomassie brilliant blue R-250 or by Western blot analysis using a rabbit anti-*EC* antiserum or a murine monoclonal antibody (no. 105) rai against native *ECor*L. The blots were visualized with go anti-rabbit or anti-mouse antibodies coupled to alkaline pho phatase, followed by incubation with nitro-blue tetrazoli (0.3 mg/ml) and bromochloroindolyl phosphate (0.15 mg/m in *N*,*N*-dimethylformamide [16]. To obtain larger amounts r*ECor*L, the *E. coli* BL21(DE3) cells containing pET-*ECor* were grown as for small-scale expression except that 1 l m dium was used and, after addition of IPTG to a final conce tration of 0.4 mM, the temperature was shifted to 25°C an the culture was further incubated overnight at the same te perature.

Preparation of inclusion bodies

Cells from an overnight 1-l culture were collected by centrifugation, suspended in 100 ml 0.15 M NaCl, 50 mM Tris/HCl pH 8 (NaCl/Tris buffer), containing 0.3 mg/m lysozyme, and left overnight at −20°C. Lysis of the bacteri cells was done by thawing, adding 0.5 ml 1 M $MgCl_2$ an DNase I to a final concentration of 7 µg/ml and sonicatin for 5 min with a Branson B-12 sonicator. The insoluble ma terial was then collected by centrifugation at 8000 × *g* in Sorvall RC-5B centrifuge, and washed twice in 1 l NaCl/Tris buffer with 1% Triton X-100 in order to solubilize and remove membranes and membrane-bound proteins. The washings were done by stirring the insoluble material with a magnetic stirrer for 1 h at room temperature followed by centrifugation as above. The insoluble material obtained after the last washing was kept at −20°C until used for solubilization.

Solubilization and refolding

r*ECor*L inclusion bodies were solubilized with 10 ml 6 M urea in 10 mM 3-(cyclohexylamino)-1-propanesulfonate (Caps) pH 10.5. The volume of the buffer-containing urea was increased until the absorbance of the solution at 280 nm was below 0.5. Refolding was done by slowly diluting the urea solution tenfold with 10 mM Caps pH 10.5, followed by addition of 0.01 vol. 1 M Tris pH 8, and further adjustment of the pH to 7.5 using concentrated HCl. The resulting solution was then concentrated by ultrafiltration to about 50 ml using a Millipore (Bedford, MA) Minitan acrylic ultrafiltration system and was dialyzed extensively against 10 mM Tris/HCl pH 7.5, containing 1 mM $CaCl_2$ and 1 mM $MnCl_2$. A precipitate of the non-refolded lectin, which was always formed during this procedure, was removed by centrifugation (8000 × *g*, 15 min in the cold) before storage of the lectin solution at −20°C. Prior to any characterization test, the lectin solution was further concentrated (using an Amicon ultrafiltration stirred cell with a PM-10 membrane) to approximately 1 mg/ml, estimated by absorbance at 280 nm on the assumption that a solution containing 1 mg/ml of *ECor*L has the same absorbance as *E. cristagalli* lectin, namely 1.53 [9].

SDS/PAGE and detection of proteins in gels

Denaturing PAGE was performed according to Laemmli [17] using 12% separation gels and 2.5% stacking gels.

Analytical molecular sieve chromatography

The molecular masses of the native and recombinant *ECor*L were determined by gel filtration using a Sephadex G-

a rabbit anti-ECo
ody (no. 105) rai
visualized with g
led to alkaline pho
tro-blue tetrazoli
sphate (0.15 mg/
in larger amounts
taining pET-ECor
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were collected b
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150 column (1.5×90 cm) equilibrated with 10 mM Tris/HCl pH 7.5, 0.15 M NaCl; elution was with the same buffer. The column was calibrated with the following proteins of known molecular mass: bovine serum albumin (67 kDa), ovalbumin (43 kDa), bovine chymotrypsinogen (25 kDa) and bovine ribonuclease A (13.7 kDa).

Gel diffusion

Gel diffusion was done according to Ouchterlony [18] except that 1% Triton X-100 was added to 1% agarose in phosphate-buffered saline [15 mM, pH 7.2).

Hemagglutinating activity

The hemagglutinating activity of the lectin was assayed by the serial dilution method in microtiter plates [19] except that 25 µl lectin solution and 25 µl 4% suspension of human-type O erythrocytes were used. A unit of activity is defined as the lowest concentration of lectin giving visible agglutination. The inhibitory activity of sugars was measured by mixing serial dilutions of the sugar with 4 hemagglutinating units of the lectin before the addition of erythrocytes and determining the lowest concentration giving full inhibition of agglutination.

N-Terminal sequence determination

Native and recombinant *ECor*L (50 µg) were run on a 12% SDS gel, and electrophoretically transferred to a polyvinylidene difluoride membrane. The membrane was stained with 0.1% Coomassie brilliant blue R250, bands corresponding to the lectin were cut out from the membrane [20] and sequenced on a gas-phase Applied Biosystem automatic sequencer, model 470A.

Carbohydrate determination

Total neutral carbohydrate content was determined by the phenol/H_2SO_4 method [21] using mannose as reference sugar.

RESULTS

Construction of the expression vector pET-*ECor*L

For expression of *ECor*L in *E. coli*, the vector pET-3d was used [15, 22]. This plasmid is a derivative of pBR322 with a strong promoter, an efficient ribosome binding site and a translation initiation region all derived from gene 10 of phage T7 (which codes for a major capsid protein of the phage). The initiation ATG codon of gene 10 is located in a unique *Nco*I site and a unique *Bam*HI site is located upstream of gene 10 transcription termination region [15]. In order to introduce the cDNA coding sequence of the complete mature *ECor*L [11], an *Nco*I site was created immediately before codon GTG at position 103–105 which, in this sequence, codes for Val27, the first amino acid of the mature lectin [23]. This *Nco*I site also introduces an ATG preceding Val27 and will code for N-terminal methionine in the recombinant lectin.

Fig. 1 shows the creation of the *Nco*I site using PCR with a 5′ end primer (CAAAGTTAACCATGGTGA) at positions 90–107 containing the necessary mutations (bold letters) and another primer (ACCATTGTTGCAGGTGT) at positions 916–932 of the 3′ end of the *ECor*L cDNA; plEcl-C [11] was the target DNA of the PCR reaction. A fragment of 800 bp was obtained and subsequently subcloned in a blunt-end fashion into a pUC-8 vector previously digested wtih *Hinc*

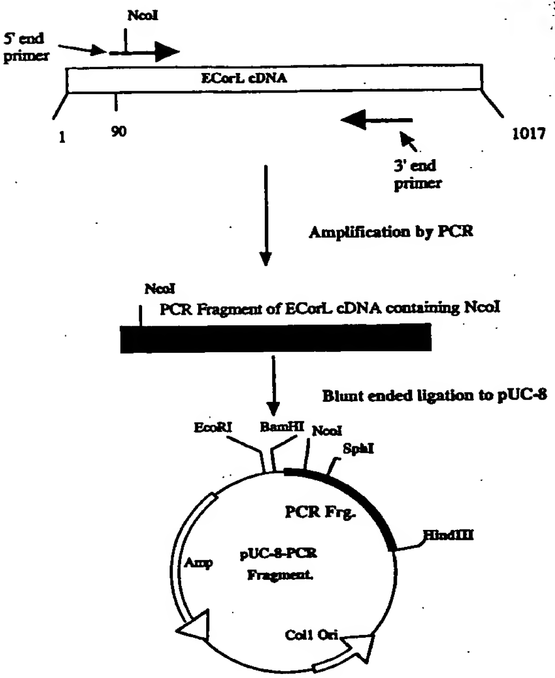
NcoI
5' end primer
ECorL cDNA
1
90
1017
3' end primer
Amplification by PCR
NcoI
PCR Fragment of ECorL cDNA containing NcoI
Blunt ended ligation to pUC-8
EcoRI
BamHI
NcoI
SphI
PCR Frg.
HindIII
Amp
pUC-8-PCR Fragment.
Col1 Ori

Fig. 1. Creation of *Nco*I site on *ECor*L cDNA with PCR.

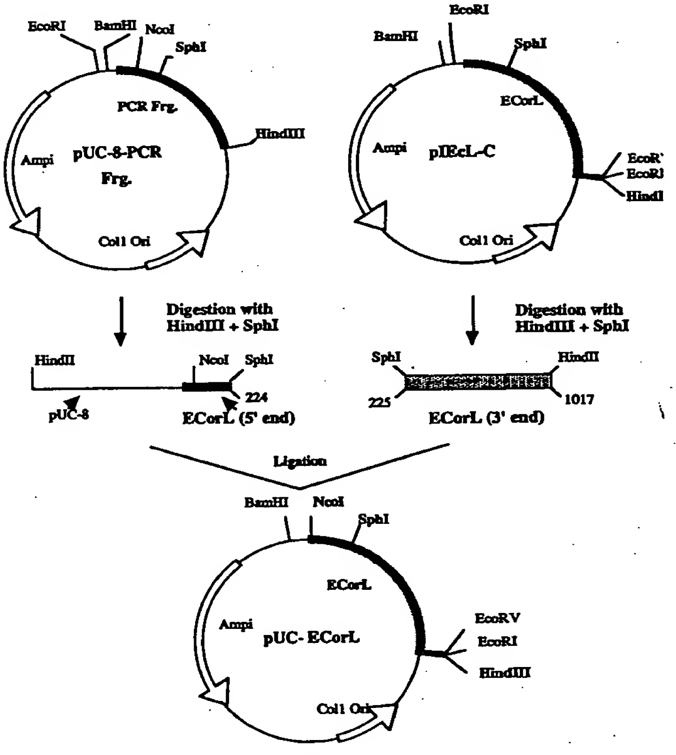
EcoRI
BamHI
NcoI
SphI
PCR Frg.
HindIII
Ampi
pUC-8-PCR Frg.
Col1 Ori
EcoRI
BamHI
SphI
ECorL
Ampi
pIEcL-C
EcoR
EcoRI
HindI
Col1 Ori
Digestion with HindIII + SphI
Digestion with HindIII + SphI
HindII
NcoI
SphI
224
pUC-8
ECorL (5' end)
SphI
HindII
225
1017
ECorL (3' end)
Ligation
BamHI
NcoI
SphI
ECorL
Ampi
pUC- ECorL
EcoRV
EcoRI
HindIII
Col1 Ori

Fig. 2. Construction of pUC-*ECor*L.

In order to reconstruct the entire coding sequence of t lectin, an internal *Sph*I site, located at position 224 of t nucleotide sequence, was used as shown in Fig. 2. The pU

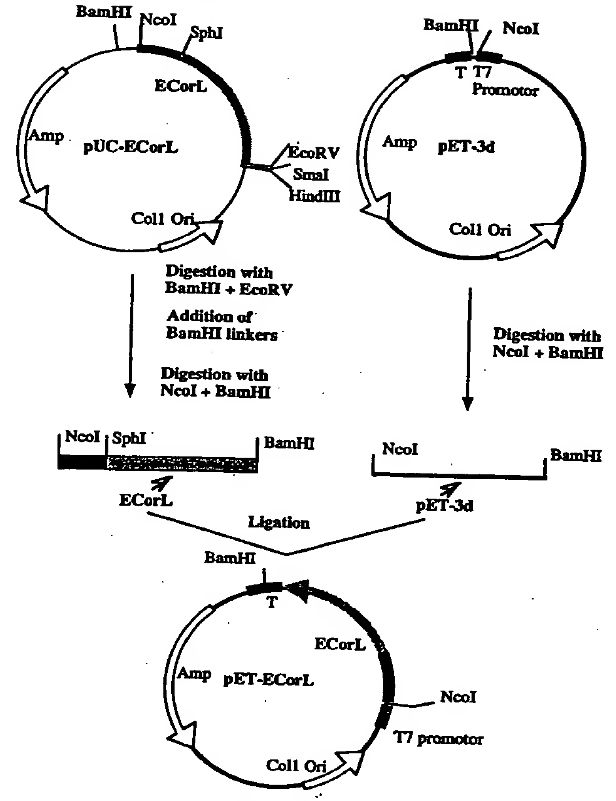

Fig. 3. Construction of pET-*ECor*L.

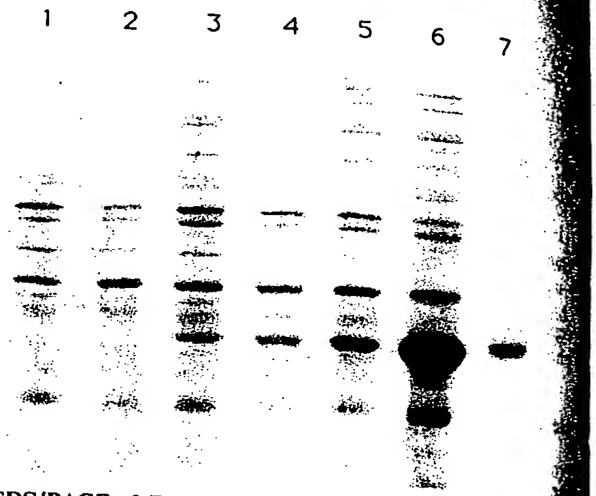

Fig. 4. SDS/PAGE of *E. coli* BL21 carrying pET-*ECor*L. Cells w grown at 37°C until late-log phase and then induced by the additi of IPTG to 0.4 mM. At different time points, 1-ml aliquots of culture were taken, cells collected by centrifugation and suspended 100 μl SDS/PAGE sample buffer. SDS/PAGE was performed by Laemmli procedure [17] with 12% gels. Proteins were visualized Coomassie brilliant blue. Lane 1, BL21 cells with pET-3d alone; la 2, cells containing pET-*ECor*L before the addition of IPTG; lanes 3– 6, cells containing pET-*ECor*L induced with IPTG for 1, 2, 3 and 18 h, respectively; lane 7, *ECor*L.

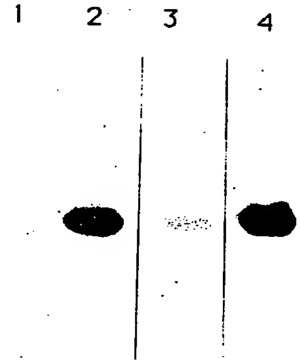

Fig. 5. Western blot analysis of *E. coli* BL21 cells carrying pET-*ECor*L. Crude lysates from bacteria carrying pET-*ECor*L were size fractionated on a 12% SDS/PAGE and transferred to a nitrocellulose membrane. Blots were probed with polyclonal rabbit anti-*ECor*L antiserum or with a mouse monoclonal antibody raised against the lectin. Lane 1, BL21 cells with pET-3d alone probed with polyclonal antiserum; lanes 2 and 3, cells containing pET-*ECor*L induced with IPTG for 3 h at 37°C, probed with polyclonal antiserum and a monoclonal antibody, respectively; lane 4, native *ECor*L probed with polyclonal antiserum.

8 vector containing the PCR fragment was digested with *Sph*I and *Hin*dIII, generating two fragments, the first containing the vector sequences plus 124 bp of the 5′ end of *ECor*L cDNA which contains the *Nco*I mutation and the other (not shown in Fig. 2), a *Sph*I–*Hin*dIII fragment of 641 bp containing most of the 3′ coding sequence of the lectin. pIEcl-C was digested with *Sph*I and *Hin*dIII releasing a fragment that contains all the coding sequence of *ECor*L at the 3′ end of the *Sph*I site (nucleotides 225–868) and a 3′ nontranslated sequence (nucleotides 869–1017); the first fragment obtained from the pUC-8-PCR vector and the 3′ end fragment from the pIEcl-C vector were ligated, creating a pUC-8 plasmid, designated as pUC-*ECor*L, which contained the entire coding sequence of the mature *ECor*L with the *Nco*I mutation before the N-terminal valine of the mature lectin. Sequence determination of the 5′ end of this construct, up to the *Sph*I site, confirmed the *Nco*I mutation and ensured that no other undesired mutations had been produced.

The *ECor*L cDNA containing the *Nco*I site was then subcloned into the expression vector pET-3d as shown in Fig. 3. pUC-*ECor*L was digested with *Bam*HI and *Eco*RV, releasing the entire *ECor*L coding fragment and the non-coding sequences. *Bam*HI linkers were ligated to the blunt-ended *Eco*RV end of the fragment. This linked fragment was then digested with *Nco*I and *Bam*HI and the product was ligated to the pET-3d plasmid previously opened by digestion with *Nco*I and *Bam*HI, to generate a recombinant plasmid designated as pET-*ECor*L.

Expression of *ECor*L in *E. coli*

Fig. 4 shows the results of SDS/PAGE analysis of lysates of *E. coli* BL21(DE3), containing pET-*ECor*L, before and after induction with IPTG at 37°C. For comparison, analysis of a lysate of the parent strain containing pET-3d is also included. A band that migrates slightly faster than the native lectin is visible in the lysates after 1 h of induction. Western blot analysis of the cell lysates revealed that this protein band reacts with rabbit anti-*ECor*L antiserum as well as with a murine monoclonal antibody raised against the native lectin (Fig. 5). The amount of r*ECor*L increased (up to 150 mg protein/l culture) when the temperature during the induction was lowered from 37°C to 25°C (Fig. 6).

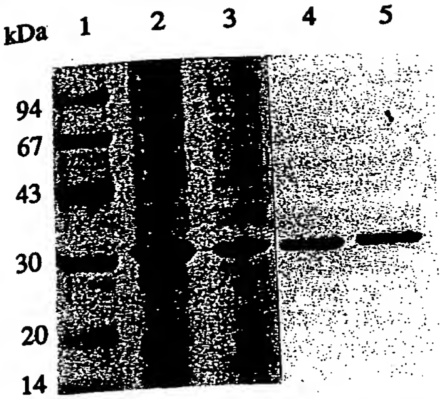

Fig. 6. Isolation of r*ECor*L from *E. coli*. Protein samples were analyzed by electrophoresis on 12% SDS/PAGE and stained with Coomassie blue. Lane 1, molecular mass markers: phosphorylase *b* 94 kDa, albumin 67 kDa, ovalbumin 43 kDa, carbonic anhydrase 30 kDa, trypsin inhibitor 20.1 kDa, α-lactalbumin 14.4 kDa. Lanes 2 and 3, bacterial lysates of BL21 cells induced with IPTG overnight at 25°C and 37°C, respectively; lane 4, recombinant *ECor*L purified from inclusion bodies; lane 5, native *ECor*L.

Purification and refolding of recombinant *ECor*L

A small amount of soluble r*ECor*L was found in the culture medium and isolated by immunoaffinity chromatography on an anti-*ECor*L column. However, the yield was so low (less than 0.1 mg/l) that it was not studied in detail. The insoluble fraction of the cell lysate contained most of the r*ECor*L in the form of inclusion bodies and was recovered by centrifugation. Contaminating membrane-bound proteins, fragmented cell walls and membranes were removed by several washings in a buffer containing Triton X-100 and lysozyme. This procedure yielded a pellet of protein aggregates, which, upon analysis by SDS/PAGE, proved to contain almost exclusively the recombinant lectin (data not shown). Soluble, active protein was recovered by dissolving the inclusion bodies in a large volume of 6 M urea, and removal of the denaturant under alkaline conditions (pH 10.5) as described under Methods. After the refolding step, the protein preparation was analyzed by SDS/PAGE and proved to contain only the recombinant lectin (Fig. 6). Typically, 4–7 mg active r*ECor*L from 1 l culture was obtained, which represented a yield of approximately 3–4% of the total r*ECor*L present in inclusion bodies.

Characterization of r*ECor*L

SDS/PAGE

As shown in Fig. 6, r*ECor*L has an apparent subunit molecular mass of close to 28 kDa on SDS/PAGE, slightly lower than the native protein. This is consistent with the absence in the r*ECor*L of the carbohydrate moiety present in *ECor*L (one heptasaccharide, molecular mass 1171 Da/subunit [7, 24]). Indeed, analysis for neutral sugar confirmed that the recombinant lectin contains less than 0.7% carbohydrate, compared to about 6% in native *ECor*L.

Molecular mass determination

The molecular mass of the recombinant protein, as estimated by gel filtration, was around 56 kDa, compared to 60 kDa of the native protein, showing that both proteins form dimers in solution. The slightly lower molecular mass of the recombinant protein can be accounted for by the absence of covalently bound carbohydrate.

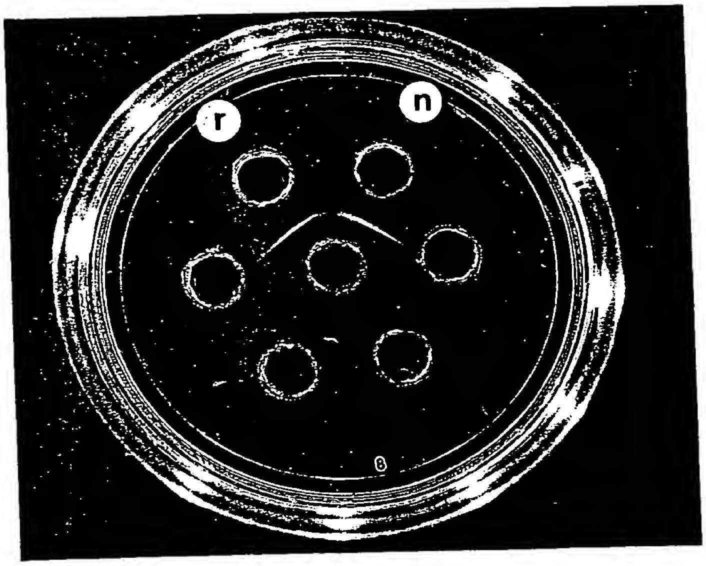

Fig. 7. Ouchterlony double-diffusion analysis of recombinant and native *ECor*L with anti-*ECor*L polyclonal antibodies. Wells: r, r*ECor*L, 10 μg; n, *ECor*L, 10 μg; center, 10 μl undiluted rabbit anti-*ECor*L antiserum.

Immunological assays

Blots of the purified recombinant lectin reacted with the rabbit anti-*ECor*L antibodies as well as with six monoclonal antibodies prepared against the native *ECor*L (data not shown). In addition, on Ouchterlony gel immunodiffusion analysis using polyclonal antibodies to the native *ECor*L, the recombinant and the native lectin developed lines of identity with each other (Fig. 7).

N-terminal sequence

In r*ECor*L 70% of the lectin molecules have the N-terminal sequence Met-Val-Glu-Thr-Ile, whereas 30% have Val-Glu-Thr-Ile-Ser, which is the same as the N-terminal pentapeptide of the native lectin [23].

Hemagglutination assays

The minimal hemagglutinating concentration of r*ECor*L was the same as that of the native protein (3 μg/ml and 3.3 μg/ml, respectively). The results of inhibition of hemagglutination are given in Fig. 8. The hemagglutinating activity of both lectins was 2–4 times more sensitive to inhibition by *N*-acetylgalactosamine than by galactose. Methyl *β*-*N*-dansylgalactosaminide was a powerful inhibitor, being 256 and 512 times stronger than galactose for *ECor*L and r*ECor*L, respectively. The two lectins were equally sensitive to *N*-acetyllactosamine (16 times more than to galactose) and no inhibition was observed by 250 mM glucose or mannose.

DISCUSSION

In this paper, we describe the construction of an expression vector containing the entire coding sequence of the mature

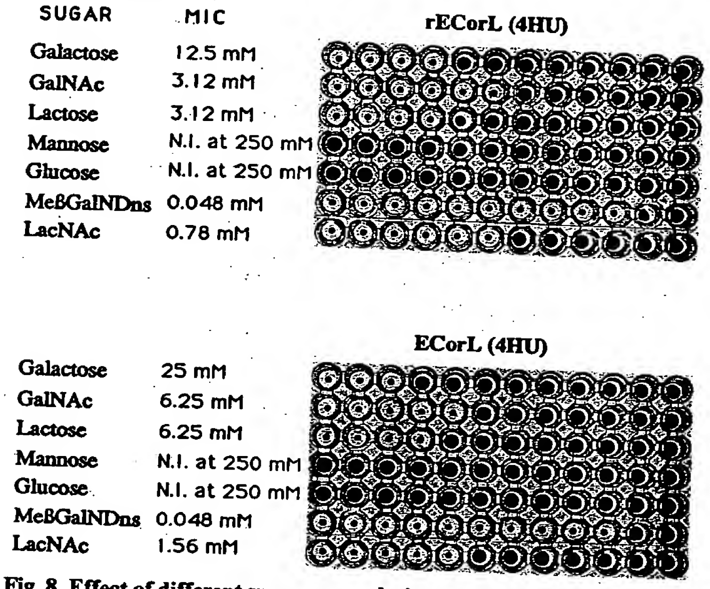

Fig. 8. Effect of different sugars on agglutination of human erythrocytes by native and recombinant *ECorL*. Each well contains 4 hemagglutinating units of the lectin, plus serial double dilutions of the inhibitor. The sugar used and the minimal concentration at which it is inhibitory (MIC) are indicated on the left. MeβGalNDns, methyl β-*N*-dansylgalactosaminide; LacNAc, *N*-acetyllactosamine; N.I., not inhibitory.

*ECor*L, its expression in *E. coli*, and the isolation and characterization of a functionally active recombinant lectin. Although small quantities of the lectin were found in soluble active form in the culture medium, the bulk was in the form of inclusion bodies from which active protein could be recovered after solubilization with urea and refolding in alkaline conditions. Accumulation of recombinant proteins as insoluble aggregates in *E. coli* is a common phenomenon [25]. To obtain an active protein from these aggregates usually necessitates the use of strong denaturing agents, such as urea or guanidinium hydrochloride, followed by refolding of the protein to its native conformation [25]. This last step requires somewhat different conditions, which are unique to each protein and have to be determined empirically. In our case, the alkaline pH of the buffer used for diluting out the urea, and a concentration of protein of less than 0.35 mg/ml ($A_{280} < 0.5$) before dilution, were critical for the recovery of active protein; when neutral buffers or higher protein concentrations were used, all the recombinant protein precipitated immediately.

Several legume lectins have been successfully expressed in *E. coli* [26–28]. Recombinant pea lectin was found to be expressed in the form of inclusion bodies, which were solubilized with guanidinium hydrochloride but, in contrast to our case, the refolding step did not require the use of alkaline conditions and the yield of active protein was higher than ours (between 10–20%) [26].

The recombinant *ECor*L is very similar in its properties to the native protein as shown by a number of criteria: gel-filtration chromatography showed that the recombinant lectin exists in solution as a dimer; Ouchterlony double-diffusion analysis with polyclonal antibodies and Western blot analysis with several monoclonal antibodies showed that both lectins are immunologically indistinguishable. There are, however, minor differences between recombinant *ECor*L and the native protein: r*ECor*L is not glycosylated and, as a result, its molecular mass is slightly lower than that of the native lectin. A 70% of r*ECor*L molecules contain an additional methio at the N-terminal. Our results indicate that these differe do not affect the hemagglutinating activity of the protein; its carbohydrate specificity. Indeed, both the native and recombinant lectins are inhibited by the same spectrum carbohydrates without any significant differences, eit qualitatively or quantitatively. Taking into consideration low accuracy of the hemagglutination assay, our data are good agreement with the relative inhibitory activities repor for *ECor*L [8, 10, 29] and in the range found for ot *Erythrina* lectins [3, 8]. This point is of crucial importance further structure/function relationship studies on the lecti carbohydrate binding site. The finding that the carbohydr moiety of *ECor*L has no effect on its binding properties su ports other reports showing that carbohydrate-free combinant lectins are as active as the native ones [28]. Th the activity of glycoprotein lectins is independent of th covalently bound carbohydrate was proposed by us some years ago [30].

We now have a system that allows us to alter individu amino acids in the binding site of *ECor*L and in this w to study their effect on the sugar specificity of the protei According to currently proposed models, based on X-ray cry tallography of complexes of sugars with *ECor*L and oth lectins [7, 31–34], the ligand-binding specificity display by these proteins is attained by the coupling of adjustab topological parameters, such as the outline of the β-turns an shape of the variable residues lining the binding pocket, wi a conserved constellation of residues (Asp89, Gly107, Phe1 and Asn133 in *ECor*L) involved directly in hydrogen bon and hydrophobic interactions with the bound sugar. Alte ation of selected amino acid residues and sequences withi the carbohydrate-binding pocket of r*ECor*L, by site-directe mutagenesis, will provide us with valuable information for better understanding of the carbohydrate specificity of legum lectins, and hopefully of lectins from other sources as well.

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